

L-ARGININE AND L-PHENYLALANINE BASED POLY (ESTER AMIDE)S,
THEIR SYNTHESIS, CHARACTERIZATION, FORMULATIONS AND
APPLICATIONS AS GENE DELIVERY VECTORS AND TISSUE ENGINEERING
SCAFFOLDS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Jun Wu

January 2011

© 2011 Jun Wu

L-ARGININE AND L-PHENYLALANINE BASED POLY (ESTER AMIDE)S,
THEIR SYNTHESIS, CHARACTERIZATION, FORMULATIONS AND
APPLICATIONS AS GENE DELIVERY VECTORS AND TISSUE ENGINEERING
SCAFFOLDS

Jun Wu, Ph.D.

Cornell University 2011

A family of water soluble and positively charged L-arginine based poly (ester amide)s (Arg-PEAs) was synthesized by solution polycondensation. These biodegradable Arg-PEAs consist of 3 nontoxic building blocks: L-arginine, diols and dicarboxylic acids. The Arg-PEAs were prepared by the reaction of tetra-p-toluenesulfonic acids salts of bis-(L-arginine) α , ω -alkylene diesters and di-p-nitrophenyl esters of dicarboxylic acids. Optimal conditions of the monomers and polymers synthesis were investigated, and the monomers and Arg-PEAs were chemically characterized. Arg-PEAs were found to have good solubility in water and many other polar solvents. . Arg-PEAs were evaluated by many biological assays for the gene delivery applications. Structure-function relationship of the Arg-PEAs revealed that changing the number of methylene groups in the diol or/and diacid segment could finely tune the hydrophobic and cationic properties of the Arg-PEAs, and then affect the gene delivery efficiency. MTT assay showed that all the prepared Arg-PEAs and Arg-PEA/DNA complexes were non-toxic to the cell lines even at very large doses. Some of Arg-PEAs showed comparable or higher transfection efficiency than the commercial transfection agents, Superfect[®] and Lipofectamine2000[®].

Based on the above results, a new generation of Arg-PEAs, oligoethylene glycols and L-arginine based poly (ether ester amide)s (Arg-PEEAs) were developed. The

new Arg-PEEAs had more flexible chain due to the introduction of oligoethylene glycols. Structure-function relationship of the Arg-PEEAs was intensively studied. MTT assay showed that all the and Arg-PEEA/DNA complexes were non-toxic to the cell lines, primary cells and stem cells even at very large doses. The Arg-PEEAs expanded the gene transfection from cell lines to primary cells/stem cells, and showed comparable or higher transfection efficiency than the commercial transfection agents, Superfect[®] and Lipofectamine2000[®].

Arg-PEAs with double bond functionality (Arg-UPEAs) could be photo-crosslinked with Pluronic- diacrylate (Pluronic-DA) to form cationic hybrid hydrogels. The physicochemical and mechanical properties of the hybrid hydrogels were studied. The fibroblast and endothelial cells were cultured on the hybrid hydrogel surface and inside the hydrogel, respectively. The results indicated that the introduction of Arg-UPEAs could significantly increase the cell attachment performance on hydrogel surface and viability inside the hydrogel.

Some new L-phenylalanine based poly (ester amide)s (Phe-PEA) or derivatives were developed as the coating materials causing low inflammatory response. One example is the block copolymer of Phe-PEA and poly (ϵ -caprolactone) (PCL) [PEA-*b*-PCL], another example is the L-Arginine and L-phenylalanine based hybrid poly (ester amide)s (Arg-Phe-PEAs). The new biomaterials were characterized and studied the cellular responses, such as cell attachment and macrophage inflammatory response. The results indicated that they could promote the cell attachment and cause very low inflammatory response.

BIOGRAPHICAL SKETCH

Jun Wu was born in Jiangsu, P. R. China in 1978, the son of father Zhonghuang Wu, mother Shengjuan Li and brother of younger sister Ping Li. He stayed in the hometown, Gaoyou, for the childhood. The hometown weather in China is very much like that of Ithaca's in the United States, where he spent 5 years on Ph.D. work. After graduated from Gaoyou high school in hometown, he went to Nanjing University for his undergraduate study in the Department of Chemistry, and received a bachelor's degree in 2000. With more interests in investigating the mystery of materials, he went to US for graduate study in 2002. After obtaining a master degree from chemistry department of SUNY at Stony Brook, he went to Cornell University for Ph.D. study under the guidance of Drs. Chih-Chang Chu, Ruth Collins and Lawrence Bonassar in the area of biomedical engineering. His research project at Cornell focused on the new biomaterials synthesis, fabrication and application. At Cornell, he never felt lonely by being alone in a foreign country because he met many new friends in US.

To my family

ACKNOWLEDGMENTS

I am deeply thankful to my advisor, professor Chih-chang Chu, for his dedicated guidance and constant support all through my graduate study at Cornell. Professor Chih-chang Chu gave me the chance and trusted me to move forward on many interesting research projects. He gave me the discipline for focus and the freedom to be inspired by what science has to offer. My research ideas and experimental work would not go anywhere without his open mind and penetrating insight. I would also thank to my other committee members: Professor Roth Collins and Professor Lawrence Bonassar. Their guidance, specialties and support have greatly improved the quality of my research.

Collaboration between different fields and schools are critical to a successful research project. I greatly appreciate Professor Bo Liu at Cornell Weill Medical College, who supplied all her lab resources to me without any reservation and trained me all the related biological techniques for the gene delivery project. I thank Professor Cynthia Reinhart-King for sharing her lab facilities with me for my tissue engineering project. I also want to thank many other professors from Cornell University and other universities for the collaborations and discussions.

I want to thank all the group members of the Chu lab. They made my time and research in Ithaca much easier and happy.

Last but not the least, I would like to thank my family. Their support has been endless and I could not have done this without them.

TABLE OF CONTENTS

BIOGRAPHICAL SKETCH	iii
DEDICATION	iv
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	xvi
LIST OF TABLES	xxii
LIST OF ABBREVIATIONS	xxiv
CHAPTER 1 INTRODUCTION: POLYCATIONS AS NON-VIRAL GENE	
DELIVERY VECTORS	1
1.A Abstract	2
1.B Gene Therapy	2
1.C Gene Delivery Vectors	3
1.D In Vitro Gene Delivery Pathway for Cationic Polymers	4
1.E Natural Polymers as Non-Viral Gene Delivery Vectors	8
1.E.1 Chitosan	8
1.E.2 Dextran and Cyclodextrin	10
1.F Synthetic Polymers as Non viral Gene Delivery Vectors	13
1.F.1 Non biodegradable Synthetic Polymers	13
1.F.1.a Polyethylenimine (PEI)	13
1.F.1.b Polymethacrylate	16
1.F.2 Biodegradable Synthetic Polymers	18
1.F.2.a Poly (L-lysine) (PLL)	18
1.F.2.b Poly (glycoamidoamine)	20
1.F.2.c Poly (amido amine) (PAA)	21

1.F.2.d Poly(4-hydroxy-L-proline)	22
1.F.2.e Poly[R-(4-aminobutyl)-L-glycolic acid] (PAGA)	22
1.F.2.f Poly(amino ester)	23
1.F.2.g Phosphorus Based Polymers	23
1.G Conclusion	24
CHAPTER 2 SYNTHESIS AND CHARACTERIZATION OF IONIC	
CHARGED WATER SOLUBLE ARGININE BASED POLY (ESTER AMIDE)	49
2.A Abstract	50
2.B Introduction	50
2.C Experimental	54
2.C.1 Materials	54
2.C.2 Measurement Methods	55
2.C.3 Synthesis of Monomers and Polymers	56
2.C.3.a Synthesis of Di-p-nitrophenyl Ester of Dicarboxylic Acids (I)	56
2.C.3.b Synthesis of Tetra-p-toluenesulfonic Acid Salt of Bis(L-arginine) Alkylene Diesters (II)	60
2.C.3.c Synthesis of Arg-PEAs (III) by Solution Polycondensation of I and II Monomers	61
2.C.4 Cell Culture and Cytotoxicity	62
2.C.5 Gel Retardation Assay	63

2.C.6 Fabrication of Arg-PEA/ Polyethylene Glycol Diacrylate (PEGDA) Hybrid Hydrogel	63
2.D Results and Discussion	64
2.D.1 Synthesis of Monomers	65
1.D.2 Synthesis of Arg-PEA Polymers and Their Properties	66
2.D.3 Molecular Weight and Charge Density of Arg-PEAs	73
2.D.4 X-Ray Diffraction	76
2.D.5 Cytotoxicity of Arg-PEAs by MTT Assay	76
2.D.6 Analysis of Polymer/Nucleic Acid Complexes by Gel Retardation Assay	77
2.D.7 Fabrication of Arg-PEA/PEGDA Hybrid Hydrogel	78
2.E Conclusion	82
CHAPTER 3 WATER SOLUBLE ARGININE POLY (ESTER AMIDE) AS GENE DELIVERY VECTOR AND INVESTIGATION OF STRUCTURE-FUNCTION RELATIONSHIP	86
3.A Abstract	87
3.B Introduction	88
3.C Experimental	93
3.C.1 Materials	93
3.C.2 Synthesis of Monomers and Polymers	94
3.C.3 Gel Retardation Assay	96
3.C.4 Cell Culture	97
3.C.5 Preparation of Plasmid DNA and Complexes of Arg-PEA/DNA for Zeta Potential Measurement and Transfection Study	97
3.C.6 Zeta Potential Measurements for Arg-PEA/DNA Complexes	98
3.C.7 Gene Transfection and Luciferase Assay	99
3.C.8 Green Fluorescence Protein (GFP) Assay	100

3.C.9 Evaluation of Cytotoxicity of the Arg-PEA/DNA complexes	101
3.C.10 Statistics	101
3.D Results and Discussions	102
3.D.1 Synthesis and Physicochemical Characterization of Arg-PEAs	102
3.D.2 Gel Retardation Assay	106
3.D.3 Transfection Efficiency	112
3.D.4 GFP Expression	114
3.D.5 Zeta Potential Measurements for DNA/Arg-PEA Complex	115
3.D.6 Cytotoxicity of Arg-PEA/DNA Complex by MTT Assay	124
3.E Conclusion	125
CHAPTER 4 ACVANCED GENERATION OF AMINO ACID-BASED POLY (ESTER AMIDE)S AS NON-VIRAL GENE DELIVERY VECTOR FOR PRIMARY AND STEM CELLS AND STRUCTURE-FUNCTION STUDY	136
4.A Abstract	137
4.B Introduction	138
4.C Experimental	142
4.C.1 Materials	142
4.C.2 Synthesis of Monomers and Polymers	144
4.C.3 Characterizations	148
4.C.4 Electrophoresis Assay	149
4.C.5 Cell Culture	152
4.C.6 Preparation of Plasmid DNA and Complexes of Arg-PEEA/DNA	152
4.C.7 Zeta Potential and Particle Size Measurements for Arg-PEEA/DNA Complexes	153
4.C.8 Gene Transfection and Luciferase Assay	154
4.C.9 Green Fluorescence Protein (GFP) Assay	156

4.C.10 Evaluation of Cytotoxicity of the Arg-PEEA/DNA Complexes	156
4.C.11 Statistics	157
4.D Results and Discussions	157
4.D.1 Synthesis and Physicochemical Characterization of Arg-PEEAs	158
4.D.1.a Synthesis of monomers	158
4.D.1.b Synthesis of Arg-PEEA Polymers	161
4.D.2 Gel Retardation Assay	166
4.D.3 Transfection Efficiency	171
4.D.4 GFP Expression	178
4.D.5 Zeta Potential and Particle Size Measurements for Arg-PEEA/DNA Complex	179
4.D.6 Cytotoxicity of Arg-PEEA/DNA Complex by MTT Assay	182
4.E Conclusion	188
CHAPTER 5 ARGININE-BASED POLY (ESTER AMIDE)S AND PLURONIC DIACRYLATE CATIONIC HYDROGEL FOR TISSUE ENGINEERING AND DRUG DELIVERY APPLICATIONS	195
5.A Abstract	196
5.B Introduction	197
5.C Experimental	201
5.C.1 Materials	201
5.C.2 Synthesis of Hydrogel Precursors	202
5.C.2.a Synthesis of Pluronic-Diacrylate (Pluronic-DA)	202
5.C.2.b Synthesis of Unsaturated Arginine Poly (ester amide) (Arg-UPEA)	203
5.C.3 Fabrication of Arg-UPEA/Pluronic-DA Hybrid Hydrogels	207
5.C.4 Measurements	208
5.C.5 Hydrogel Swelling Ratio & Swelling Kinetics	210

5.C.6 Compressive Modulus Measurement by Dynamic Mechanical Analyzer (DMA)	211
5.C.7 Cell Culture Study	211
5.C.8 Cytotoxicity Evaluation of Precursors by MTT Assay	212
5.C.9 Cell Attachment and Proliferation on Arg-UPEA/F127-DA Hybrid Hydrogels Surface	213
5.C.10 Cell Viability inside Arg-UPEA/F127-DA Hybrid Hydrogels	214
5.C.11 Controlled Release of Hydralazine (Apresoline) via Arg-UPEA/F127-DA Hybrid Hydrogels	214
5.C.12 Statistics	216
5.D Results and Discussions	216
5.D.1 Preparation and Characterization of Precursors	216
5.D.1.a Synthesis and Characterization of Pluronic-DA	216
5.D.1.b Synthesis and Characterization of Arg-UPEAs	217
5.D.2 Fabrication and Characterization of Arg-UPEA/Pluronic-DA Hybrid Hydrogels	221
5.D.3 Equilibrated Swelling Ratio & Swelling Kinetics of Arg-UPEA/Pluronic-DA in DI Water and Buffers	223
5.D.4 Mechanical Property (Compressive Modulus) of Arg-UPEA/F127-DA Hybrid Hydrogels	229
5.D.5 Interior Morphology (SEM) of Arg-UPEA/F127-DA Hydrogels	230
5.D.6 Cytotoxicity of Hydrogel Precursors	231
5.D.7 Cell Attachment and Proliferation on Hydrogel Surfaces	235
5.D.8 Cell Viability inside Arg-UPEA/F127-DA Hybrid Hydrogels	239
5.D.9 Controlled Release of Hydralazine (Apresoline) via Arg-UPEA/F127-DA Hybrid Hydrogels	243

5.E Conclusions	244
CHAPTER 6 POLY (ESTER AMIDE)-b-POLY (ϵ -CAPROLACTONE): SYNTHESIS, CHARACTERIZATION, FORMULATION, AND IN VITRO CELLULAR RESPONSE	
6.A Abstract	253
6.B Introduction	254
6.C Experimental	256
6.C.1 Materials	256
6.C.2 Synthesis of monomers and polymers	257
6.C.2.a Synthesis of Monomers: Di-p-nitrophenyl Ester of Dicarboxylic Acids (I) and Di-p-toluenesulfonic Acid salt of Bis (L-Phenylalanine) Alkylene Diesters (II)	259
6.C.2.b Synthesis of PEA (III) by Solution Condensation of (I) and (II)	259
6.C.3 Synthesis of Phe-PEA-b-PCL	260
6.C.4 Characterization	261
6.C.5 Formulation of PEA-b-PCL into Microspheres	263
6.C.6 Formulation of Phe-PEA-b-PCL into Microfibers by Electrospun Method	263
6.C.7 In Vitro Enzymatic Biodegradation of PEA-b-PCL	264
6.C.8 Cell Culture	265
6.C.9 Cell Attachment and Proliferation on PEA-b-PCL	265
6.C.10 In Vitro Measurement of Inflammatory Response of PEA-b-PCL	266
6.C.11 Statistics	267
6.D Results and Discussion	267
6.D.1 Synthesis and Characterization of PEAs with Functional End Groups	267
6.D.2 Synthesis and Characterization of PEA-b-PCL	269
6.D.3 Solubility	272
6.D.4 Static Contact Angle	273

6.D.5 Thermal Property of PEA-b-PCLs	276
6.D.6 Formulation of PEA-b-PCL into Microspheres and Electrospun Fibers	277
6.D.7 In vitro Enzymatic Biodegradation of PEA-b-PCL	280
6.D.8 Cell Attachment and Proliferation Assays	281
6.D.9 In Vitro Inflammatory Response of PEA-b-PCL	283
6.E Conclusion	287
CHAPTER 7 WATER INSOLUBLE IONIC CHARGED BIODEGRADABLE POLY (ESTER AMIDE)S: SYNTHESIS, CHARACTERIAZTION AND IN VITRO CELLULAR RESPONSE	
7.A Abstract	297
7.B Introduction	298
7.C Experimental	300
7.C.1 Materials	300
7.C.2 Synthesis of Monomers and Polymers	301
7.C.2.a Synthesis of Monomers	301
7.C.2.b Synthesis of Arg-Phe-PEA (III) by Solution Polycondensation of (I) and (II) Monomers	301
7.C.3 Measurements	303
7.C.4 In vitro Enzymatic Biodegradation of Arg-Phe-PEA	307
7.C.5 Cell Culture	307
7.C.6 Cell Attachment and Proliferation Assay on Arg-Phe-PEA Coatings	308
7.C.7 In Vitro Measurement of Inflammatory Response of Arg-Phe-PEA	309
7.C.8 Statistics	310
7.D Results and Discussion	310
7.D.1 Synthesis and Characterization of PEAs	310
7.D.2 Solubility	311

7.D.3 Static Contact Angle	311
7.D.4 Thermal Property of Arg-Phe-PEAs	315
7.D.5 In vitro Enzymatic Biodegradation of Arg-Phe-PEA	317
7.D.6 Cell Attachment and Proliferation Assays	321
7.D.7 In vitro Inflammatory Response of Arg-Phe-PEAs	323
7.E Conclusion	327
APPENDICES	333
Appendix 1: Chapter 3	333
A1.1 Transfection and cytotoxicity evaluation of SMC A10 and RSMC primary cell	333
A1.1.1 MTT assay for cytotoxicity evaluation	333
A1.1.2 Firefly luciferase assay for transfection efficiency evaluation	335
A1.1.3 GFP assay	338
A1.1.4 Cell morphology (48 h, 10X) for RSMC primary cells	339
A1.2 DNA delivery by UArg-PEA	339
A1.2.1 Firefly luciferase assay for transfection and cytotoxicity efficiency evaluation of UArg-PEA	339
Appendix 2: Chapter 4	343
A2.1 Firefly luciferase assay for transfection efficiency evaluation of A10 SMC cell lines	343
A2.2 Transfection and cytotoxicity evaluation of SVEC4-10 endothelial cell	345
A2.2.1 Firefly luciferase assay for transfection efficiency evaluation	345
A2.2.2 GFP assay	348
A2.2.3 MTT assay	349
A2.3 Transfection and cytotoxicity evaluation of RAW 264.7 macrophages	349
A2.3.1 Firefly luciferase assay for transfection efficiency evaluation	349

A2.3.2 GFP assay	352
A2.3.3 MTT assay	352
A2.3.4 Cell morphology (48 h, 10X) for RAW 264.7 macrophages cells	355
A2.4 Transfection and cytotoxicity evaluation of rat aortic fibroblast primary cell	355
A2.4.1 GFP assay	357
A2.4.2 Cell morphology (48 h, 10X) for rat aortic fibroblast primary cell	358
A2.5 Inflammation assay for Arg-PEA/DNA complex	358
Appendix 3: Chapter 5	360
A3.1 BAEC and fibroblast cell attachment on hydrogel surface	360
Appendix 4: Chapter 6	364
A4.1 Materials	364
A4.2 Synthesis of PEAs	364
A4.3 Material characterization	365
A4.4 Results and discussion	365

LIST OF FIGURES

Figure 1.1 Examples of Amine Groups for Gene Delivery	7
Figure 1.2 Chemical Structure of Chitosan	8
Figure 1.3 Chemical Structure of Chitosan-g-PEG	9
Figure 1.4 Chemical Structure of Dextran	11
Figure 1.5 Chemical Structure of β -cyclodextrin	11
Figure 1.6 Chemical Structure of Linear PEI	14
Figure 1.7 Chemical Structure of Poly (methacrylate)	17
Figure 1.8 Chemical Structure of Poly (L-lysine)	19
Figure 1.9 Chemical Structure of Poly (glycoamidoamine)	21
Figure 1.10 Chemical Structure of Poly (amido amine)	22
Figure 2.1 Chemical structure of poly (ester amide)	53
Figure 2.2 Chemical structure of Arg-PEAs	54
Figure 2.3 Synthesis of monomer I	57
Figure 2.4 Synthesis of monomer II	58
Figure 2.5 Synthesis of Arg-PEAs	59
Figure 2.6 FTIR spectra of monomer II	69
Figure 2.7 FTIR spectra of Arg-PEAs	70
Figure 2.8 HNMR spectra of 2-Arg-2-S	71
Figure 2.9 Solubility of Arg-PEAs in distilled water	75
Figure 2.10 X-ray diffraction diagram of Arg-PEAs	79
Figure 2.11 Cytotoxicity tests of Arg-PEAs by MTT assay	80
Figure 2.12 DNA condensation ability of Arg-PEA	81
Figure 2.13 Hydrogel image of 2-U-Arg-2-S/PEG-DA	81
Figure 3.1 General Chemical Structure of an Amino Acid-based Poly (ester amide)	91
Figure 3.2 Chemical structure of Arg-PEAs	91

Figure 3.3 Synthesis of Monomer I	94
Figure 3.4 Synthesis of Monomer II	94
Figure 3.5 Synthesis of Arg-PEAs from monomers I and II	95
Figure 3.6 Arg-PEA solubility in distilled water	104
Figure 3.7 Gel retardation assay of Arg-PEAs	110
Figure 3.8 The minimum weight ratio (WR) of Arg-PEA to DNA	111
Figure 3.9 Transfection efficiency of Arg-PEA/DNA complexes	116
Figure 3.10 Effect of x and y material parameters of Arg-PEAs on the transfection efficiency	117
Figure 3.11 The effect of x and y material parameters of Arg-PEAs on their weight ratios of Arg-PEA to DNA	119
Figure 3.12 Fluorescence microscopic view of the GFP expression from transfected SMC A10 cells	120
Figure 3.13 Zeta potential measurements of Arg-PEA/DNA complex	121
Figure 3.14 Cytotoxicity of Arg-PEA/DNA complexes by MTT assay	122
Figure 3.15 RSMC cell morphology	123
Figure 4.1 General Chemical Structure of Arginine Based Poly (ester amide)	140
Figure 4.2 Chemical structure of Arg-PEEAs	142
Figure 4.3 Monomer I	144
Figure 4.4 Monomer IIa	144
Figure 4.5 Monomer IIb	145
Figure 4.6 ^1H -NMR spectra of 2-Arg-2E-S	164
Figure 4.7 Effect of methylene chain length (x) of Arg-PEEAs on their condensation ability to DNA	169
Figure 4.8 Effect of oligoethylene glycol chain length (y) of Arg-PEEAs on the condensation ability to DNA	170

Figure 4.9 Effects of type of Arg-PEEAs and weight ratio of Arg-PEEAs to DNA on transfection efficiency	173
Figure 4.10 Effects of rigidity/flexibility of Arg-PEA backbone and weight ratio of Arg-PEAs to DNA on transfection efficiency	174
Figure 4.11 GFP expression of transfected cells under fluorescence microscope	180
Figure 4.12 Zeta potential of 2-Arg-6E-Cl/DNA complex	181
Figure 4.13 Particle size measurements of 2-Arg-6E-Cl/DNA complex	183
Figure 4.14 MTT cytotoxicity of 4 types of Arg-PEEA	184
Figure 4.15 Human umbilical vein endothelial cells (HUVEC) and rat mesenchymal stem cells (MSC) morphology	185
Figure 4.16 Rat mesenchymal stem cells (MSC) morphology	186
Figure 5.1 Chemical Structure of Unsaturated Arg-PEA	200
Figure 5.2 Chemical Structure of Hydralazine	201
Figure 5.3 Synthesis of Pluronic-DA	204
Figure 5.4 Synthesis of Monomer I	205
Figure 5.5 Synthesis of Monomer II	205
Figure 5.6 Synthesis of Arg-UPEAs	206
Figure 5.7 Image of hybrid hydrogel of 2-U-Arg-2-S/F127-DA	223
Figure 5.8 Swelling kinetics of Arg-UPEA/Pluronic-DA hydrogels in DI water	225
Figure 5.9 Equilibrated swelling ratio of Arg-UPEA/Pluronic-DA hydrogels in PBS	227
Figure 5.10 the equilibrium swelling ratios of the hybrid hydrogels in aqueous solution	229
Figure 5.11 SEM images of Arg-UPEA/F127-DA hybrid hydrogels	232
Figure 5.12 Representative micrographs of fibroblast cells after 48 hr culture	233
Figure 5.13 Fibroblast cell viability (%) after incubation in various amount of F127-	

DA and 2-U-Arg-2-S	234
Figure 5.14 Representative micrographs of fibroblast cells	237
Figure 5.15 MTT Assay for fibroblast cells	238
Figure 5.16 live-dead assay for BAEC cells encapsulated in the pure F127-DA hydrogel	240
Figure 5.17 Controlled Release of Hydralazine Chlorine Salt via Arg-UPEA/F127-DA Hydrogel	241
Figure 6.1 Chemical Structure of Poly (ester amide)	256
Figure 6.2 Synthesis of Monomer I	258
Figure 6.3 Synthesis of Monomer II	258
Figure 6.4 Synthesis of PEAs via solution polycondensation	258
Figure 6.5 Synthesis of PEA- <i>b</i> -PCL from the ring-opening polymerization	261
Figure 6.6 ¹ H-NMR spectrum of 8-Phe-4- <i>b</i> -PCL	275
Figure 6.7 the relationship of T _m	278
Figure 6.8 SEM image of PEA- <i>b</i> -PCL microspheres	279
Figure 6.9 SEM image of PEA- <i>b</i> -PCL electrospun fibers	279
Figure 6.10 Effect of PEA to PCL feed ratio on the enzymatic biodegradation of the PEA- <i>b</i> -PCL copolymer	281
Figure 6.11 Microscopic Image of attached BAEC on the polymer coatings	285
Figure 6.12 Proliferation assay of BAEC on polymer coatings	286
Figure 6.13 In vitro Measurement of Inflammatory Response of Polymers	287
Figure 7.1 Chemical structure of poly (ester amide)	300
Figure 7.2 Synthesis of di- <i>p</i> -nitrophenyl ester of dicarboxylic acids monomer	304
Figure 7.3 Synthesis of monomer II	305
Figure 7.4 Synthesis of Arg-Phe-PEAs by a solution polycondensation	306
Figure 7.5 ¹ HNMR spectrum of 8-Arg-6-S-8-Phe-4-20%	313

Figure 7.6 T_g of Arg-Phe-PEA copolymers	316
Figure 7.7 α -chymotrypsin (0.2 mg/mL) enzymatic degradation of Arg-Phe-PEA	320
Figure 7.8 SEM images of 8-Arg-4-S-8-Phe-4-20% polymer film	321
Figure 7.9 Microscopic image of attached BAEC on the polymer coatings	324
Figure 7.10 Proliferation assay of BAEC on polymer coatings	325
Figure 7.11 in vitro measurement of inflammatory response of polymers	326
Figure 1 Cytotoxicity evaluation of Arg-PEA/DNA complexes by MTT assay	335
Figure 2 SMC A10 transfection efficiency of Arg-PEA/DNA complexes	337
Figure 3 GFP assay for SMC A10 cells	338
Figure 4 RSMC cell morphology	339
Figure 5 Firefly luciferase assay for <i>SMC A10</i> cells (UArg-PEA/DNA)	342
Figure 6 Firefly luciferase assay for <i>SMC A10</i> cells	345
Figure 7 Firefly luciferase assay for <i>SVEC4-10</i> endothelial cells	347
Figure 8 GFP assay for <i>SVEC4-10</i> endothelial cells	348
Figure 9 MTT assay for Arg-PEA/DNA for <i>SVEC4-10</i> endothelial cells	349
Figure 10 Firefly luciferase assay for RAW 264.7 macrophages cell	351
Figure 11 GFP assay for RAW 264.7 macrophages cell	352
Figure 12 MTT assay for Arg-PEA/DNA for RAW macrophages	354
Figure 13 RAW 264.7 macrophages cell morphology	355
Figure 14 GFP transfection efficiency of rat aortic fibroblast primary cell	357
Figure 15 Rat aortic fibroblast primary cell morphology cell morphology	358
Figure 16 Nitrite production from RAW macrophages	359
Figure 17 BAEC cell morphology on collagen treated hydrogel surface	361
Figure 18 BAEC cell morphology on collagen treated hydrogel surface	362
Figure 19 Human Detroit 539 fibroblast cell morphology on hydrogel surface	363
Figure 20 M_n -Time curve of polymerization of 4-Phe-4	366

Figure 21 M_n -Time curve of polymerization of 8-Phe-4	367
Figure 22 M_n -Time curve of polymerization of 8-Phe-4	368
Figure 23 M_n -Time curve of polymerization of 8-Phe-4	368
Figure 24 M_n -Time curve of polymerization of 8-Phe-4-b-PCL	369
Figure 25 M_n -Time curve of polymerization of 8-Phe-4-b-PCL	370

LIST OF TABLES

Table 2.1 Arg- PEAs prepared by different combination of monomers	62
Table 2.2 Physical and Thermal Characteristics of Arg-PEAs	68
Table 2.3 Solubility of Arg-PEAs in various solvents	74
Table 2.4 MW of some Arg-PEAs	75
Table 3.1 Arg-PEAs (x-Arg-y-S) prepared	96
Table 3.2 Arg-PEA Properties	107
Table 3.3 Molecular Weight of Arg-PEAs	108
Table 4.1 List of prepared <i>p</i> -toluenesulfonic acid salt of L-arginine	150
Table 4.2 Arg-PEEAs (x-Arg-yEG-z)	151
Table 4.3 Water solubility and reduced viscosity	167-8
Table 4.4 Relative transfection efficiency of Arg-PEEAs	175
Table 5.1 Chemical and Physical Properties of Arg-UPEAs	219
Table 5.2 Arg-UPEA/F127-DA Hybrid Hydrogels and Their Physicochemical Properties	224
Table 5.3 LC-MS Test of Released Hydralazine	244
Table 6.1 Phe-PEAs prepared by different combination of monomers	260
Table 6.2 Relationship between <i>r</i> and end groups of Phe-PEAs	270
Table 6.3 Information of some prepared Phe-PEAs	271
Table 6.4 Information of some PEA- <i>b</i> -PCLs	274
Table 6.5 Solubility of PEA- <i>b</i> -PCLs	276
Table 6.6 Static contact angle of polymers	276
Table 6.7 T_m and T_g of polymers	278
Table 7.1 Synthesized Arg-Phe-PEAs	304
Table 7.2 Solubility of Arg-Phe-PEA	314
Table 7.3 Static contact angle of polymers	319

LIST OF ABBREVIATION

Arg: L-Arginine

DA: diacrylate

DMF: dimethylformamide

DMSO: dimethyl sulfoxide

DSC: differential scanning calorimetry

FTIR: Fourier transform infrared

Leu: leucine

M_n: number average molecular weight

M_w: weight average molecular weight

NA: di-*p*-Nitrophenyl Adipate

NF: di-*p*-Nitrophenyl Fumarate

NS: di-*p*- Nitrophenyl Sebacate

NSu: di-*p*-Nitrophenyl Succate

PDI: polydispersity

PEA: poly (ester amide)

PEG: poly (ethylene glycol)

PEI: polyethylenimine

Phe: phenylalanine

PLL: poly (L-lysine)

SF: superfect

T_g: glass transition temperature

T_m: melting point temperature

THF: tetrahydrofuran

CHAPTER 1
INTRODUCTION: POLYCATIONS AS NON-VIRAL GENE DELIVERY
VECTORS

Jun Wu¹, Chih-Chang Chu^{1,2}

¹ Department of Biomedical Engineering, Cornell University, Ithaca, NY 14853-4401,
USA

² Department of Fiber Science and Apparel Design, Cornell University, Ithaca, NY
14853-4401, USA

1.A Abstract

In order to improve the gene therapy efficiency, new gene delivery systems have recently been fast developed¹⁻⁹. Among them, the synthetic delivery systems, especially cationic polymer based systems, have aroused great interests from scientist and clinicians due to their versatile property choices and clinical safety¹⁻⁹. Many reports indicated that the cationic properties and chemical structure of the polymers have very important effects on DNA/vector complex size and stability, DNA uptake, stability of DNA in the endosome and cytoplasm and the targeting of DNA to the cell nucleus¹⁻⁹. The deep understanding of cationic polymers' structure-function relationship would help to elucidate the details and mechanisms for DNA delivery and stability within cells. However, few review articles systematically addressed this structure-function issue. This review chapter would go over and discuss the current status of polycations as non-viral gene delivery vectors, especially focus on how the modification of polycations' structure affect the polymer property, then affect the gene delivery efficiency. The knowledge would help to design new polycations with high transfection efficiency and low cytotoxicity simultaneously.

1.B Gene Therapy

In the early 1970s, gene therapy was first defined as the treatment of human disease by transferring the genetic material into specific cells of the patients¹⁻¹¹. During the past several decades, gene therapy has obtained significant attention as a potential method for treating genetic diseases and cancers¹⁻¹¹. With the fast growing of biotechnology, gene therapy has been developed rapidly and led to the first genetic treatment of patients under approved FDA protocols in 1990¹⁻¹¹. From that, thousands

of gene therapy clinical trials have been approved and operated worldwide¹⁻¹¹. However, the successful rate of gene therapy was not very encouraging¹⁻¹³. A successful delivery system must be able to effectively transport DNA across the plasma membrane, through the intracellular environment, and finally into the nucleus without damage¹⁻¹¹. Based on the reported gene trial results, one of the key limitations was that there had not been safe, efficient and controllable methods for gene delivery¹⁻¹³.

1.C Gene Delivery Vectors

Currently the research efforts for gene delivery are focused on the following areas¹⁻¹³: 1, obtaining effective delivery vectors that compact and protect oligonucleotides from the nucleases degradation in the blood or cytosol; 2, the delivery vectors should be biocompatible, causing low cytotoxicity and inflammation response. Normally, gene delivery vectors could be divided into viral vector and non-viral vector.

For the viral vectors, the advantages and disadvantages have been well documented¹⁻¹³. Because of the specialized structures evolved after million years, viral delivery systems, including retroviruses and adenoviruses, have exhibited pretty high delivery efficiency of DNA and RNA to numerous cells⁵. However, the limitations brought by viral vector systems, including payload capacity, toxicity and immunogenicity, targeting of specific cell types, production and packaging, recombination, and production cost, restricted their clinical applications and encouraged the investigations of non-viral gene delivery vectors⁵.

Therefore, non-viral gene delivery systems, especially synthetic DNA delivery systems, have greatly aroused the interests from both researchers and clinicians¹⁻¹³. For the non-viral synthetic gene delivery vectors, the majority of them, furthermore, could be divided into three sub categories: 1, cationic lipids/liposomes; 2, cationic dendrimers; 3, cationic polymers. All of them have shown the strong gene delivery capability. Among them, the cationic polymers aroused highest interests because of their versatile choices of polymer structure, charge property, functional groups, molecular weight and other physicochemical properties¹⁻¹³.

1.D In Vitro Gene Delivery Pathway for Cationic Polymers

Good biocompatibility, high payload capacity and easiness for large-scale production make the non-viral vectors, especially cationic polymers, increasingly attractive for gene therapy¹⁻¹¹. However, unlike viral systems that have been evolved to overcome cellular barriers and immune defense, most of the reported non-viral gene delivery vectors showed relative inefficient transfection and cytotoxicity due to the numerous cellular obstacles¹⁻¹¹. Therefore, identifying the details for each barrier of the delivery pathway would help to overcome the barriers and improve gene delivery efficiency. For the in vitro gene delivery pathway of cationic polymers, though some steps have been explained clearly, the majority steps and details of the pathway and barriers are still unclear and need further investigations¹⁻¹¹. The commonly agreed gene delivery pathway for cationic polymers could be divided into the following steps⁵: (A) DNA/polycation complex formation; (B) uptake and endocytosis; (C) escape from endosome or degradation (endosome and lysosome); (D) intracellular release or degradation (cytosol); (E) nuclear targeting, entry and expression.

The first step of the pathway is polymer/DNA complex formation⁵. By electrostatic interaction, the positively charged polycations condense negatively charged DNA and form polyelectrolyte complex. The key factors for this step are the stability and size of the complex particle in the solutions⁵. Normally, there would be excessive positive charge on the complex particle surface to keep the particle stable and facilitate the interaction with the cellular membrane. For a successful DNA delivery, the reports suggested that it would be in the range of 50-250 nm. And the aggregation of complexes would significantly reduce the gene delivery efficiency⁵.

In the second step, the complexes are taken up by cells through endocytosis⁵. Before the endocytosis, the DNA/polymer complex need attach to the cell membrane by electrostatic interaction or receptor/ligand (R/L) interaction⁵. Endocytosis is the process by which cells absorb molecules from outside the cell by engulfing them with their cell membrane and it is a multistep process involving binding, internalization, formation of endosomes, fusion with lysosomes, and lysis. The enzymes and low pH within endosomes and lysosomes usually cause degradation of entrapped DNA and associated complexes.

So the efficiency of gene delivery is related with destabilization and escaping from endosomes, which is the 3rd step of the gene delivery pathway⁵. Methods/mechanisms to enhance polycation/DNA complex early release from endosome have been actively explored. One of the escaping mechanisms is called “proton sponge effect”, which was caused by cationic polymers with buffering capability that promote endosome osmotic swelling, disruption of the endosome membrane and intracellular release of DNA.

After escaping from the endosome, the DNA need move through the cytosol toward the nucleus and the movement is probably controlled by diffusion, which is slow⁵. During this process, DNA must be protected from the cytoplasm nucleases. Before or after entering the nucleus, the DNA must be released from the complexes. For this step, only complexes with intermediate stability will gain the maximal gene expression because stable complexes may restrict DNA release and unstable complexes would cause rapid DNA degradation. The liberation rate and percentage of DNA from polycations is directly related with the polymer structural property. The final nucleus entry is to occur through nuclear pores (~10 nm in diameter) or during cell division⁵. For this nuclear targeting step, the knowledge about nuclear targeting is still relatively unclear and the understanding/exploiting nuclear targeting should greatly help to improve the DNA delivery efficiency.

Based on the above discussions, there following are major barriers for the non-viral gene delivery pathway through polycations⁵: low uptake across the cell membrane, inadequate release and stability of DNA, and lack of nuclear targeting. In addition to these major barriers, several other issues also need to be overcome, including optimization of DNA condensation, size of DNA complexes, cell targeting and cytotoxicity⁵.

Overall, the process and mechanism for polycations gene delivery are very complicated and many steps/barriers affect the success of gene delivery⁵. Since most of the key steps/barriers have direct or indirect relationships with the polycations' properties, which is determined by polycations' functional groups and structure, the investigation and elucidation of the polymer structure-function relationship is very important for polycation based gene delivery.

Almost all of the polycations' functional groups for gene delivery are the amine based groups, which are functional groups that contain basic nitrogen atoms. Depending on how many of the hydrogen atoms are replaced, amines could be divided into three different basic classes: primary amine, secondary amine and tertiary amine. Most of the amine based cationic groups, including the amine derivatives with complicated structure, such as azole family, could be looked as the different combinations of the three basic types of amines. For the amines in the amides part, the amines are attached to carbonyl group and could not show the cationic property, so they would not be considered as the functional group for DNA condensing.

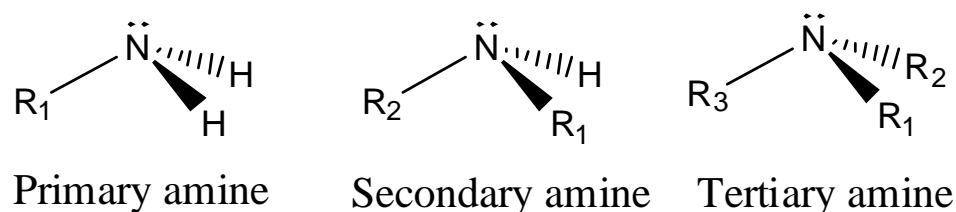


Figure 1.1 Examples of Amine Groups for Gene Delivery

This introduction chapter reviews the polycation non-viral DNA delivery systems, highlighting the typical systems and focusing on how the polymer structure and functional amine groups affect the DNA transfection efficiency, cytotoxicity and delivery mechanism. The examples and discussions would focus on applying different methods to enhance DNA penetration of the plasma membrane, approaches for optimizing protection and intracellular release of DNA, and ways of improving DNA targeting to the nucleus. In the aspects of tuning the polymer structure and other properties, the following are some general principles for the chemical modifications of

polycations: 1, by introducing hydrophilic or hydrophobic segments, such as PEG (hydrophilic) and polyester (hydrophobic), amphiphilic polymer structure could be obtained. The polymer solubility and polymer/DNA complex stability would be significantly affected; 2, by introducing functional groups with buffering capability, such as imidazole group and PEI, the proton sponge effect would be obtained; 3, by introducing peptide sequences or antibody/antigen, the complex would be able to have specific interaction with cell membrane. These modification methods could be separately applied to the specific systems or be applied with different combinations.

1.E Natural Polymers as Non-Viral Gene Delivery Vectors

1.E.1 Chitosan

Chitosan is prepared from deacetylation of chitin to have the polymer composed of D-glucosamine and *N*-acetyl- D-glucosamine subunits linked by (1, 4) glycosidic bonds^{14, 15}. The primary amine group in chitosan has a pKa value around 6.5, which made chitosan positively charged and soluble in acidic or neutral solution^{14, 15}. As the only basic polysaccharide existing in nature, the biodegradability, biocompatibility, and cationic property of chitosan has helped it become one of the most attractive non-viral gene delivery vectors¹⁴⁻¹⁷.

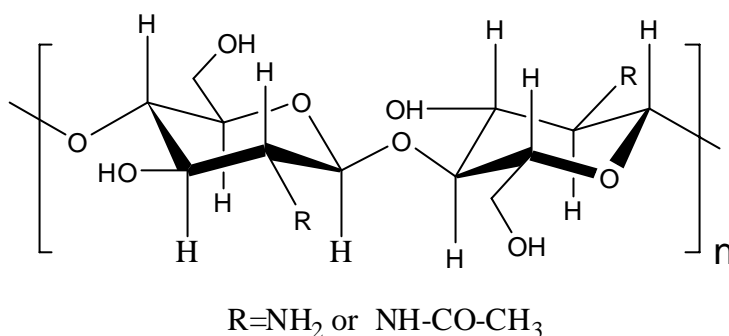


Figure 1.2 Chemical Structure of Chitosan

Since the middle of 1990s, chitosan had been widely tested for gene delivery applications¹⁴⁻¹⁸. The results indicated that molecular weight of chitosan, in a certain range, could affect the chitosan/DNA complex particle size and stability, and then influence the gene transfer efficiency¹⁹. It was found that the size of chitosan/DNA complex increases with the increasing of chitosan molecular weight and higher molecular weight chitosan forms more stable chitosan/DNA complexes^{19, 20}. In the molecular weight range of 15 kDa to 220 kDa, the transfection efficiency increases with the increasing of molecular weight of chitosan²¹. However, this molecular weight effect has not been evaluated or discussed for the chitosan with molecular weight larger than 250 kDa. To improve transfection efficiency of chitosan, numerous modifications to the polymer structure have been made^{16, 18, 20, 22-30}. For example, by conjugating chitosan with hydrophilic PEG or dextran^{14, 25, 31, 32}, the stability of chitosan/DNA complex would be improved and complex cytotoxicity would be decreased. And hydrophobic moieties, such as deoxycholic acid, stearic acid, and other alkyl chains have been conjugated to chitosan to reduce the aggregation of chitosan/DNA complex and improve the interactions between the complexes and cell surfaces^{26, 33, 34}. The transfection results showed that the alkylated chitosan derivatives perform much higher transfection efficiency than unmodified chitosan^{26, 33, 34}.

To improve the cationic properties (charge density and buffering capability) of chitosan, several methods were utilized, such as grafting chitosan with polylysine, polyethyleneimine, urocanic acid, guanidine group and quaternization of chitosan amine groups^{27, 35, 36}. These modifications resulted in improved transfection efficiency, but cause higher cytotoxicity compared to unmodified chitosan^{27, 35, 36}. Various cell-targeting ligands have been conjugated to chitosan so that chitosan/DNA

complexes could be delivered to specific cell types. Galactose, lactose, folate, and trisaccharide have also been used to improve the hepatic cell-targeting and improve the gene transfection efficiency^{24, 29, 37-39}.

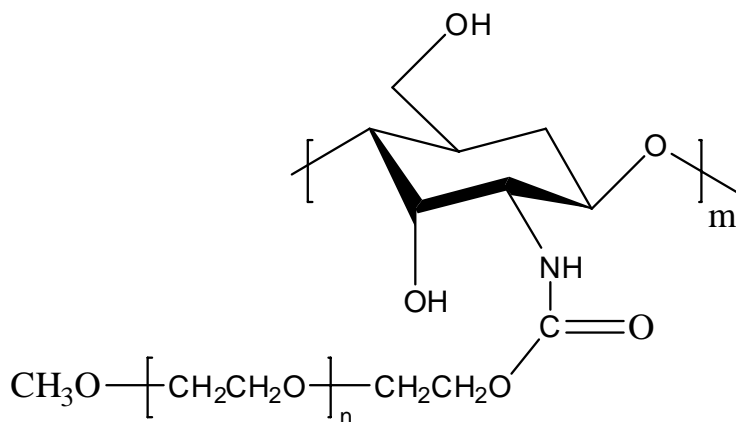


Figure 1.3 Chemical Structure of Chitosan-g-PEG

1.E.2 Dextran and Cyclodextrin

Dextran is a branched polysaccharide composed of glucose units with varying lengths and the molecular weight is ranging from 10 to 150 kDa. Because of its good biocompatibility and wide availability, dextran has aroused interest from scientists for the gene delivery applications⁴⁰⁻⁴². Since dextran itself has no amine based groups, the chemical modification is needed to introduce the positively charged amine groups to dextran⁴⁰⁻⁴². One example is diethylaminoethyl-dextran (DEAE-dextran), which has been widely used for gene transfer applications for decades⁴⁰.

Recently, dextran-g-spermine polycations have been synthesized by oxidizing dextran with potassium periodate followed by reductive amination with spermine and

the polymers showed good transfection efficiency^{41, 42}. However, the further modification, quaternization of dextran-g-spermine showed reduced transfection efficiency⁴³. The reason could be due to the hindered release of DNA from complex or the significant change of the polymer structure⁴³. Further modification of dextran-g-spermine with PEG showed improved gene transfer efficiency⁴⁴.

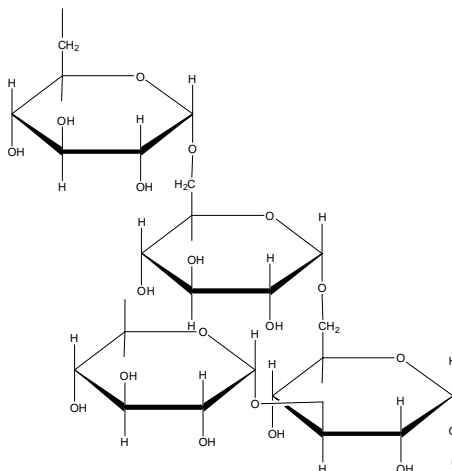


Figure 1.4 Chemical Structure of Dextran

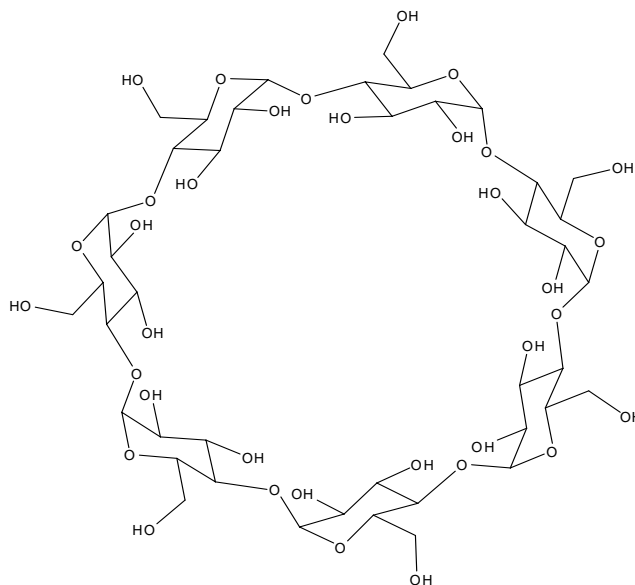


Figure 1.5 Chemical Structure of β -cyclodextrin

Another type of glucose based polysaccharide system, cyclodextrin, was also widely evaluated for gene delivery applications because of the excellent biocompatibility and plenty of applications for the drug delivery⁴⁵⁻⁵⁵. Davis et al. reported that incorporating β -cyclodextrin into cationic polymers by polymerization of a bifunctional β -cyclodextrin monomer to prepare linear β -cyclodextrin based polymers^{49, 53, 56}. Transfection results showed good gene transfer efficiency and limited toxicity⁴⁹. Further investigation showed that the length of methylene units (n) between β -cyclodextrin monomer units affects the cyclodextrin/DNA complex's transfection efficiency and cytotoxicity: longer chain lengths cause decreased charge density, and then decrease the complex cytotoxicity⁵⁰. The results indicated that for the methylene units (n) ranging from 4 to 10, lowest cytotoxicity and highest transfection efficiency were obtained for polymers with 6, 7, or 8 methylene units⁵⁰. And for n equals 10, the polymer showed highest toxicity and lowest transfection

efficiency, which could be due to the reduced polymer solubility⁵⁰. And the reduced polymer solubility means the increased hydrophobicity. Similar study of β -cyclodextrin based complex showed that increasing the hydrophilicity of the β -cyclodextrin spacer unit can reduce cytotoxicity because of the improved chain flexibility⁵⁷. However, quantitative evaluation of the hydrophobicity/hydrophilicity is still needed for this system⁵⁷.

Other chemical modifications were also applied to cyclodextrin system. The PEGylation of cationic β -cyclodextrin could help to reduce the complex aggregation⁴⁸. Peptide conjugated β -cyclodextrin polymers, such as adamantane-PEG-galactose or adamantane-PEG-transferrin conjugated polymer, have shown successful cell targeting gene transfer ability⁵⁸. Imidazole or PEI modified β -cyclodextrin complex could own buffering capacity and facilitate the endosomal escape. However the cytotoxicity of the modified cyclodextrin may be increased.^{59, 60}

Overall, for the polysaccharides based polymers as gene delivery carriers, the limitations are mainly focused on the following areas: 1, most of them have very broad molecular weight distribution. Currently there was no very effective method for this issue; 2, the sugar ring unit of the polysaccharides caused the less flexibility of polymer chain, which may affect the stability of the polymer/DNA complex. The introduction of PEG may help to improve the flexibility; 3, missing or inadequate nitrogen based functional groups for DNA condensing. Conjugating with PLL, PEI or other amine based groups/polymers would help to solve this issue, but the cytotoxicity may be increased.

1.F Synthetic Polymers as Non-viral Gene Delivery Vectors

1.F.1 Non-biodegradable Synthetic Polymers

1.F.1.a Polyethylenimine (PEI)

Polyethylenimine (PEI) is one of the most widely investigated non biodegradable cationic polymers for gene transfection^{36, 61-75}. In 1995, Behr et al reported the first test of PEI as gene carrier⁶⁴. After that, PEI and its derivatives have been widely used for gene delivery applications^{36, 61-75}. Due to the mixed primary/ secondary/tertiary amines in the polymer chain, PEI has been shown the buffering capability, which was called the “proton sponge effect”⁷⁵. Because of the high density of amines, PEI showed strong self protonation ability. At the physiological pH, there are about 80% of the amines remaining unprotonated and less than 50% nitrogens were unprotonated at a pH of 5⁶⁷. This buffering capacity allows PEI/DNA complex to avoid lysosomal trafficking and the following degradation.

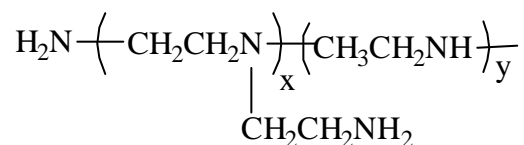


Figure 1.6 Chemical Structure of Linear PEI

For the molecular weight effect, Godbey et al. showed that transfection efficiency of PEI increases with the increasing of molecular weight in the range of 600 and 70,000 Da⁷⁶⁻⁷⁸. However, it has been proved that higher molecular weight PEI caused higher cytotoxicity⁷⁶⁻⁷⁸. The degree of branching of PEI also affects DNA complex formation and stability⁷⁹. Chemical modifications to PEI polymer structure have been applied to improve transfection efficiency^{61, 72, 80-89}. PEGylation creates a

hydrophilic exterior environment that reduces interactions of the PEI/DNA with plasma proteins^{80, 82, 84, 90}. It was found that the length and density of PEG chains conjugated to PEI have an effect on transfection efficiency. DNA could be effectively transfected when a high density of short PEG chains (550 Da) are grafted onto PEI^{81, 86}. Besides PEGylation, quaternization of amines, RGD peptides, transferrin, folate, mannose and galactose have been introduced to PEI to improve transfection efficiency and the transfection results were very positive^{69, 84, 85, 87, 90-94}.

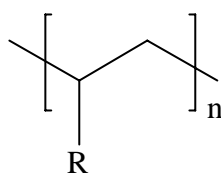
The hydrophobic effect was investigated by reacting PEI with the hydrophobic amino acids (such as alanine and leucine) or dodecyl and hexadecyl halides⁶⁹. After reaction, the PEI hydrophobicity was significantly enhanced and transfection efficiency was improved⁶⁹. Many other functional groups were used to modify PEI to improve transfection efficiency, such as linking cholesterol to the amines⁷¹. For low molecular weight branched PEI (1.8 kDa), transfection efficiency increases and toxicity is reduced with the addition of cholesterol⁷¹. Lee et al. synthesized biodegradable PEI derivatives by treatment of low molecular weight PEI (800 Da) with linkers⁹⁵. These compounds showed reduced transfection efficiency and cytotoxicity compared to 25 kDa PEI⁹⁵. In addition to disulfide linkages, PEI derivatives with ester linkages have been prepared to create biodegradable gene carriers^{61, 63, 72, 89}. Kim et al. synthesized biodegradable PEI-PEG conjugates by reacting low molecular weight PEI (600, 1200, 1800 Da) with PEG succinimidyl succinate (2000 Da)⁶¹. These PEI-PEG conjugates showed reduced cytotoxicity and improved gene transfer ability⁶¹. However, it should be pointed out that the degradation products of PEI-PEG are non biodegradable PEI and PEG. Kissel et al. incorporated PEG-polycaprolactone (PEG-PCL) grafts onto the PEI structures^{89, 96}. These polymers showed reduced cytotoxicity and some of them exhibited improved

transfection efficiency^{89, 96}. Cho et al. investigated PCL-PEI conjugates synthesized via Michael addition of PEI (600, 1200, 1800 Da) and polycaprolactone diacrylate⁶³. Results showed that these PEI derivatives exhibit significantly higher transfection efficiency compare to 25 kDa PEI⁶³. Pack et al. synthesized PEI derivatives with ester linkages by similar reactions and the new compounds showed significantly lower cytotoxicity and higher transfection efficiency than 25 kDa PEI⁹⁷. Cho et al. synthesized linear PEI-*b*-PEG using a Michael-type addition with low molecular weight PEI (423 Da) and PEG diacrylates⁷⁴. These compounds exhibited reduced cytotoxicity and improved gene transfer efficiency compared to 25 kDa PEI⁷⁴. In addition to the disulfide and ester linkages, hydrolyzable amide and imine linkages have also been investigated^{72, 98}. Kissel et al. synthesized low molecular weight L-lactic acid-*co*-succinic acid and reacted this compound with low molecular weight branched PEI (1200 Da). These amide-linked compounds exhibited reduced cytotoxicity compared to 25 kDa PEI and improved transfection efficiency compared to 1.2 kDa PEI⁷². Kim et al. synthesized imine linked PEI by treating low molecular weight branched PEI (1.8 kDa) with glutadialdehyde⁹⁸. While these structures showed reduced cytotoxicity, transfection efficiency was lower than that of 25 kDa PEI⁹⁸.

1.F.1.b Polymethacrylate

Because of the cationic charge, poly [2-(dimethylamino) ethyl methacrylate] (PDMAEMA) and its derivatives were tested as gene transfer agents⁹⁹. Evaluations of these compounds showed that the highest transfection efficiency with acceptable cytotoxicity could be obtained from the polymers when the polymer molecular weight is greater than 300 kDa¹⁰⁰. The successful in vitro transfection of PDMAEMA/DNA complex was thought to be due to the polymer's endosome destabilizing ability¹⁰¹.

Various chemical modifications have been applied to PDMAEMA to improve the transfection efficiency. Hennink et al. attempted to improve the endosome escaping capability of PDMAEMA by incorporating an additional tertiary amino group in each unit to promote the “proton sponge” effect¹⁰². However, the modified polymer showed reduced cytotoxicity and transfection efficiency¹⁰³, which could be due to the significant change of the polymer structure. Schacht et al. converted various percentages of the amine groups of PDMEMA into pyridine, imidazole, and carboxylic acid groups to improve endosomal escape. The transfection efficiency for all the modified systems were decreased, which continued to indicate that proton sponge effect may not be the only dominating effect for gene delivery and the polymer structure effect is also very important¹⁰³. Guanidinium side groups were introduced to PDMAEMA because of the strong cell membrane penetrating capability and the modified polymer showed improved transfection efficiency¹⁰⁴.



R= ester groups

Figure 1.7 Chemical Structure of Poly (methacrylate)

Hydrophobic and hydrophilic segments were incorporated to reduce the cytotoxicity of PDMAEMA^{105, 106}. For examples, the 2-(dimethylamino)ethyl methacrylate could be copolymerized with other monomers, such as methyl methacrylate (MMA), a hydrophobic monomer; *N*-vinyl-pyrrolidone (NVP), a hydrophilic monomer; and ethoxytriethylene glycol methacrylate (triEGMA), a

hydrophilic monomer¹⁰⁵. The PDMAEMA-MMA copolymer showed reduced transfection efficiency and increased cytotoxicity¹⁰⁶. The PDMAEMA-triEGMA and the PDMAEMA-NVP copolymers both showed reduced cytotoxicity and improved transfection efficiency¹⁰⁶. Polymer structure-function study showed that increased ratios of comonomer to PDMAEMA decreased cytotoxicity, but also reduced the polymer DNA condensing capability¹⁰⁶. However, these copolymers showed significant transfection efficiency improvements¹⁰⁶.

To promote cellular uptake of PDMAEMA-based polymers, many targeting agents, such as folate, hepatocyte-targeting agents (including galactose and lactose), or tumor targeting protein, have been incorporated onto the PDMAEMA and the modified PDMAEMA showed improved transfection efficiency¹⁰⁷⁻¹¹¹. In recent years, incorporation of hydrolyzable side chains to PDMAEMA polymers to obtain biodegradable PDMAEMA gene carriers has been tried^{112, 113}. For example, Hennink et al. synthesized a methacrylate-based polymer with carbonate component (pHPMA-DMAE) and the polymer showed improved gene transfer efficiency¹¹⁴.

1.F.2 Biodegradable Synthetic Polymers

1.F.2.a Poly (L-lysine) (PLL)

Poly (L-lysine) (PLL) is prepared by the ring-opening polymerization of *N*-carboxy-(*N*-benzyloxycarbonyl)-L-lysine anhydride (Lysine-NCA) using a primary amine initiator¹¹⁵. The Lysine-NCA was synthesized from the ϵ -amine protected L-lysine¹¹⁵. Since the primary ϵ -amino groups of PLL have a pKa value of 9.7, all NH₂ groups of PLL are protonated at physiological pH. In 1975, Laemmli et al reported the using of poly (L-lysine) (PLL) for DNA condensation¹¹⁶. After that, this polymer

was quickly evaluated for gene transfer tests^{35, 90, 117-130}. According to the sponge effect theory, PLL could not offer buffering capacity to help the PLL/DNA complex escape from endosome. The endosomal release of PLL/DNA complex could be improved by adding chloroquine, which raises endosomal pH to decrease DNA degradation by inhibiting lysis¹²⁵. Another modification for buffering effect is to graft the PLL with imidazole groups to, which have a p*K*_a around 6.0, providing PLL with buffering capacity¹²⁹.

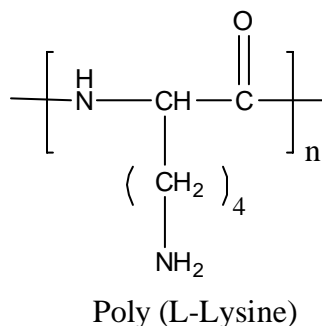


Figure 1.8 Chemical Structure of Poly (L-lysine)

For the molecular weight effect, PLL need a molecular weight higher than 3000 Da to effectively condense DNA to form stable complexes¹²². However, the high molecular weight PLL showed relatively high cytotoxicity¹¹⁹. The incorporation of imidazole groups into PLL or prepare multi-arm PLL derivatives could reduce the cytotoxicity^{129, 131}. PLL/DNA complexes were found to tend to aggregate and precipitate in the solution¹³². In order to avoid the formation of insoluble precipitates and enhance the stability of PLL/DNA complex, poly (ethylene glycol) (PEG) was introduced to PLL^{117, 119, 121, 133-135}. The complexes of PLL-*b*-PEG/DNA showed the ability to reduce the complex particle size, stabilize the particles and resistant to deoxyribonuclease I (DNase I) digestion^{121, 133}. On the other side, the polyester

segments were grafted to the PLL chain so that the hydrophobicity and hydrolysis property were introduced. Poly (lactic-*co*-glycolic acid) (PLGA) and poly (lactide-*b*-ethylene glycol) (PLA-*b*-PEG) have been successfully grafted into the PLL chain and the new polymers showed reduced cytotoxicity and significant transfection efficiency compared to PLL¹³⁵⁻¹³⁷. And histidine residues could further introduced to promote buffering capacity¹²⁹.

To overcome nonspecific cell targeting, various targeting moieties were introduced to PLL, such as apoprotein E derived peptide¹³⁸, galactose¹³⁹, lactose¹¹⁹, folate⁹⁰ and transferrin^{140, 141}. Antigen-antibody interactions could also be used to improve cell targeting. Besides PLL, poly (L-arginine) and poly (L-histidine) have also been evaluated for gene delivery applications and they showed very good cell membrane penetration capability.

1.F.2.b Poly(glycoamidoamine)

Poly (glycoamidoamine) is a polymer family with carbohydrate component along a linear amino backbone¹⁴². For example, the poly (glycoamidoamines) with D-glucaric acid as the carbohydrate moiety showed almost no cytotoxicity and significant gene transfection efficiency¹⁴³. And the transfection efficiency could be improved when increase chain length of the amine-containing monomer unit¹⁴³. Further study showed that the stereochemistry of the hydroxyl groups of the poly (glycoamidoamine) also affects the transfection efficiency by altering the stability of the complex^{144, 145}. Recent studies continued to investigate the structure-function relationships of poly (glycoamidoamines)^{59, 146} and the results showed that the higher density of amino groups promoted greater cellular uptake and the gene delivery

efficiency was related to the complex stability¹⁴⁷. Recent study also indicated that the branched poly (glycoamidoamines) were less toxic due to the decreased secondary amine density, but the gene delivery performance was decreased, too¹⁴⁸.

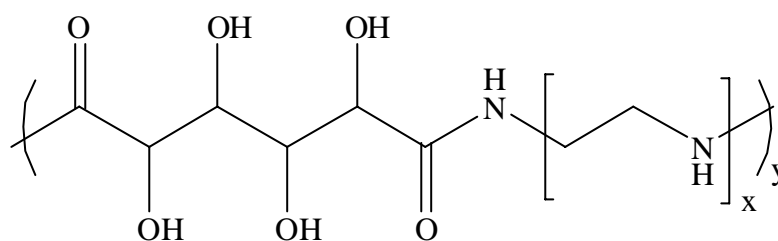


Figure 1.9 Chemical Structure of Poly (glycoamidoamine)

1.F.2.c Poly (amido amine) (PAA)

The cationic linear poly (amido amines) have been prepared by hydrogen-transfer polymerization of aliphatic primary monoamines or bis(secondary amines) and bisacrylamides to form polymer structures with amido and tertiary amino functional groups¹⁴⁹. Barbucci et al. confirmed that the protonation of PAA limit the conformational freedom of the polymer and lead to a more rigid structure^{150, 151}. The in vitro MTT assay confirmed that the PAA had lower cytotoxicity compared with high molecular weight PLL and PEI¹⁵² and the gene transfection studies of PAA showed high transfection efficiency¹⁵³⁻¹⁵⁵. PAA with disulfide linkages in the bisacryamide monomer unit to create a reducible polymeric structure have been synthesized by Engbersen et al¹⁵⁶ and these PAAs showed reduced cytotoxicity and improved gene transfer results as compared to branched 25 kDa PEI¹⁵⁶. Modifications to the side chain of these biodegradable PAA polymers showed that both hydroxyl and histidine

functional groups could further reduce the cytotoxicity and improve transfection efficiency of PAA/DNA complex^{157, 158}.

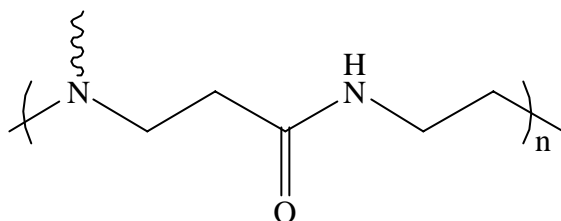


Figure 1.10 Chemical Structure of Poly (amido amine)

1.F.2.d Poly(4-hydroxy-L-proline)

Poly (4-hydroxy-L-proline) (PHP) was the first reported hydrolytically degradable cationic polymer^{130, 159}. PHP was prepared by polymerization of N-cbz-4-hydroxy- L-proline followed by deprotection¹⁶⁰. The polymer could be degraded within controlled period ranging from a few hours to 3 months¹⁵⁹. Results from the transfection studies showed that PHP could condense DNA effectively and PHP have showed good transfection efficiency and significantly reduced cytotoxicity when compared to PEI or PLL^{130, 159, 161}.

1.F.2.e Poly[R-(4-aminobutyl)-L-glycolic acid] (PAGA)

Poly[R-(4-aminobutyl)-L-glycolic acid] is a type of biodegradable polyester with similar structure of PLL. It was prepared by conversion of the R-amino group of N-cbz-L-lysine to a hydroxyl group followed by polymerization then deprotection¹⁶². This biodegradable polymer could be degraded within controlled period ranging from

a few hours to 6 months¹⁶³. In vitro gene transfer studies showed that PAGA/DNA complexes had no cytotoxicity and better transfection efficiency compared to PLL/DNA complexes^{162, 164-166}.

1.F.2.f Poly(amino ester)

Langer et al. reported the gene delivery tests using poly (β -amino esters), which could be completely degraded within a few hours without killing cells^{167, 168}. Some of the poly (β -amino esters) showed good gene transfer efficiencies compared to PEI, PLL, and Lipofectamine 2000¹⁶⁷⁻¹⁶⁹. A study of over 2300 poly (β -amino esters) prepared from hydrophobic diacrylate monomer units showed improved transfection efficiency¹⁷⁰. The structure-function relationships of these polymers revealed that molecular weight, end groups, and polymer/DNA ratio effect transfection efficiency.¹⁷⁰ Poly (β -amino esters) capped with diamine chains exhibit high transfection efficiency. However, the cytotoxicity increased a lot, which could be due to more effective DNA binding¹⁷¹. The hyperbranched poly (β -amino esters) also showed significant gene transfer capacity¹⁷²⁻¹⁷⁴.

1.F.2.g Phosphorus Based Polymers

Degradable phosphorus based polymers, such as poly (phosphazenes) (PPZ)¹⁷⁵⁻¹⁷⁷, poly (phosphoesters) (PPE)^{178, 179}, and poly (phosphoramidates) (PPA)^{178, 179}, have been widely tested for gene delivery. These polymers showed low cytotoxicity and good gene transfer capability in vitro compared to PDMAEMA¹⁷⁵⁻¹⁷⁹. Transfection efficiency of polyphosphoesters with amine terminated side chains is between PLL and PEI¹⁷⁹. Further studies showed that the DNA releasing rate was affected by the

side chain¹⁸⁰. For complexes containing alkyl side chains with terminal amines, the rate of DNA release increases with the increasing of chain length¹⁸⁰. In addition, it was shown that polymers with secondary amine-terminated alkyl side chains release DNA more quickly than the primary amine polymers¹⁸⁰. Polyphosphoramidates with spermidine side chains showed transfection efficiency better than that of PEI¹⁸¹.

1.G Conclusion

In the past decades, tremendous progresses have been made in the design and synthesis of non-viral polycation vectors for gene delivery. Some common design principles have also been widely accepted. The 1st important principle is that nearly all synthetic vectors have the positive charge necessary for electrostatic interaction with DNA. It has been widely accepted that the structure and density of the amine groups significantly affect transfection efficiency. Normally increasing the charge density of the amine groups would improve the polymer's transfection efficiency, but may cause higher cytotoxicity. The cytotoxicity could be reduced by incorporating histidine or guanidine functional groups and normally higher transfection efficiency could be achieved simultaneously. The quaternization of amine groups is another commonly used method to increase the cationic charge density. However, the effect of quaternization was not always encouraging in terms of transfection efficiency and cytotoxicity. Buffering capability is very important for obtaining high transfection efficiency, however, introducing buffering functional groups sometimes may not effectively increase the transfection ability, and the reasons for the phenomena were unclear. However, the significant changing of polymer structure may affect polycations' transfection efficiency a lot.

Introduction of hydrophilic groups, such as PEG, would improve the stability of polycation/DNA complexes, then increase the transfection efficiency and decrease the transfection cytotoxicity. Inclusion of hydrophobic groups, such as steroidal moieties, alkyl chains, and hydrophobic amino acids, would help to improve the nonspecific cellular uptake and generally result in higher transfection efficiency. Conjugating the polycation vector with a number of cell targeting agents would promote the specific cellular uptake of polycation/DNA complexes.

Overall, many efforts have been applied for the investigation of polycations structure-function relationship for gene delivery applications. However, there have not been any reported systematical and quantitative works for the polycations structure-function relationship. In order to help to develop new generation of polycation gene delivery vectors, the following chapters discussed a new polymer family, arginine based poly (ester amide), as gene delivery vectors and the structure-function relationship was intensively, systematically and quantitatively studied.

REFERENCE

1. Anderson, W. F., Human Gene-Therapy. *Science (New York, N.Y.)* **1992**, 256, (5058), 808-813.
2. Anderson, W. F., Human gene therapy. *Nature (London)* **1998**, 392, (6679, Suppl.), 25-30.
3. Anon, Gene delivery system. *Science (Washington, D. C.)* **2000**, 288, (5465), 533.
4. Banerjee, S.; Livanos, E.; Vos, J. M., Therapeutic gene delivery in human B-lymphoblastoid cells by engineered non-transforming infectious Epstein-Barr virus. *Nature medicine* **1995**, 1, (12), 1303-8.
5. Luo, D.; Saltzman, W. M., Synthetic DNA delivery systems. *Nature biotechnology* **2000**, 18, (1), 33-37.
6. Mulligan, R. C., The Basic Science of Gene-Therapy. *Science (New York, N.Y.)* **1993**, 260, (5110), 926-932.
7. Langer, R., Drug delivery: Drugs on target. *Science (Washington, DC, U. S.)* **2001**, 293, (5527), 58-59.
8. Rossi John, J.; June Carl, H.; Kohn Donald, B., Genetic therapies against HIV. *Nature biotechnology* **2007**, 25, (12), 1444-54.
9. Putnam, D., Polymers for gene delivery across length scales. *Nature Materials* **2006**, 5, (6), 439-51.
10. Friedman.T; Roblin, R., Gene Therapy for Human Genetic Disease. *Science* **1972**, 175, (4025), 949-&.
11. Wolff, J. A.; Lederberg, J., An Early History of Gene-Transfer and Therapy. *Human Gene Therapy* **1994**, 5, (4), 469-480.
12. Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S., Design and development of polymers for gene delivery. *Nature Reviews Drug Discovery* **2005**, 4, (7), 581-593.

13. Yamanouchi, D.; Wu, J.; Lazar, A. N.; Craig Kent, K.; Chu, C.-C.; Liu, B., Biodegradable arginine-based poly(ester-amide)s as non-viral gene delivery reagents. *Biomaterials* **2008**, 29, (22), 3269-3277.
14. Wu, J.; Wang, X.; Keum, J. K.; Zhou, H.; Gelfer, M.; Avila-Orta, C.-A.; Pan, H.; Chen, W.; Chiao, S.-M.; Hsiao, B. S.; Chu, B., Water soluble complexes of chitosan-g-MPEG and hyaluronic acid. *Journal of Biomedical Materials Research, Part A* **2007**, 80A, (4), 800-812.
15. Mumper, R. J.; Wang, J.; Claspell, J. M.; Rolland, A. P., Novel polymeric condensing carriers for gene delivery. *Proc. Int. Symp. Controlled Release Bioact. Mater.* **1995**, 22nd, 178-9.
16. Borchard, G., Chitosans for gene delivery. *Advanced Drug Delivery Reviews* **2001**, 52, (2), 145-150.
17. Kim, T.-H.; Jiang, H.-L.; Jere, D.; Park, I.-K.; Cho, M.-H.; Nah, J.-W.; Choi, Y.-J.; Akaike, T.; Cho, C.-S., Chemical modification of chitosan as a gene carrier in vitro and in vivo. *Progress in Polymer Science* **2007**, 32, (7), 726-753.
18. Domard, A.; Rinaudo, M., Preparation and characterization of fully deacetylated chitosan. *Int. J. Biol. Macromol.* **1983**, 5, (1), 49-52.
19. MacLaughlin, F. C.; Mumper, R. J.; Wang, J.; Tagliaferri, J. M.; Gill, I.; Hinchcliffe, M.; Rolland, A. P., Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. *Journal of Controlled Release* **1998**, 56, (1-3), 259-272.
20. Kiang, T.; Wen, J.; Lim, H. W.; Leong, K. W., The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. *Biomaterials* **2004**, 25, (22), 5293-5301.

21. Huang, M.; Fong, C.-W.; Khor, E.; Lim, L.-Y., Transfection efficiency of chitosan vectors: Effect of polymer molecular weight and degree of deacetylation. *Journal of Controlled Release* **2005**, 106, (3), 391-406.
22. Kean, T.; Roth, S.; Thanou, M., Trimethylated chitosans as non-viral gene delivery vectors: Cytotoxicity and transfection efficiency. *Journal of Controlled Release* **2005**, 103, (3), 643-653.
23. Chen, H. H.; Ho, Y.-P.; Jiang, X.; Mao, H.-Q.; Wang, T.-H.; Leong, K. W., Quantitative Comparison of Intracellular Unpacking Kinetics of Polyplexes by a Model Constructed From Quantum Dot-FRET. *Molecular Therapy* **2008**, 16, (2), 324-332.
24. Erbacher, P.; Zou, S.; Bettinger, T.; Steffan, A.-M.; Remy, J.-S., Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability. *Pharmaceutical Research* **1998**, 15, (9), 1332-1339.
25. Germershaus, O.; Mao, S.; Sitterberg, J.; Bakowsky, U.; Kissel, T., Gene delivery using chitosan, trimethyl chitosan or polyethyleneglycol-graft-trimethyl chitosan block copolymers: Establishment of structure-activity relationships in vitro. *Journal of Controlled Release* **2008**, 125, (2), 145-154.
26. Hu, F.-Q.; Zhao, M.-D.; Yuan, H.; You, J.; Du, Y.-Z.; Zeng, S., A novel chitosan oligosaccharide-stearic acid micelles for gene delivery: Properties and in vitro transfection studies. *International Journal of Pharmaceutics* **2006**, 315, (1-2), 158-166.
27. Kim, T. H.; Ihm, J. E.; Choi, Y. J.; Nah, J. W.; Cho, C. S., Efficient gene delivery by urocanic acid-modified chitosan. *Journal of Controlled Release* **2003**, 93, (3), 389-402.
28. Koeping-Hoeggard, M.; Varum, K. M.; Issa, M.; Danielsen, S.; Christensen, B. E.; Stokke, B. T.; Artursson, P., Improved chitosan-mediated gene delivery based on

easily dissociated chitosan polyplexes of highly defined chitosan oligomers. *Gene Therapy* **2004**, 11, (19), 1441-1452.

29. Murata, J.-I.; Ohya, Y.; Ouchi, T., Design of quaternary chitosan conjugate having antennary galactose residues as a gene delivery tool. *Carbohydr. Polym.* **1997**, 32, (2), 105-109.

30. Nguyen, D. N.; Green, J. J.; Chan, J. M.; Langer, R.; Anderson, D. G., Polymeric materials for gene delivery and DNA vaccination. *Advanced Materials (Weinheim, Germany)* **2009**, 21, (8), 847-867.

31. Park, I. K.; Kim, T. H.; Park, Y. H.; Shin, B. A.; Choi, E. S.; Chowdhury, E. H.; Akaike, T.; Cho, C. S., Galactosylated chitosan-graft-poly(ethylene glycol) as hepatocyte-targeting DNA carrier. *Journal of controlled release official journal of the Controlled Release Society* **2001**, 76, (3), 349-62.

32. Park, Y. K.; Park, Y. H.; Shin, B. A.; Choi, E. S.; Park, Y. R.; Akaike, T.; Cho, C. S., Galactosylated chitosan-graft-dextran as hepatocyte-targeting DNA carrier. *Journal of Controlled Release* **2000**, 69, (1), 97-108.

33. Liu, W. G.; Zhang, X.; Sun, S. J.; Sun, G. J.; Yao, K. D.; Liang, D. C.; Guo, G.; Zhang, J. Y., N-Alkylated Chitosan as a Potential Nonviral Vector for Gene Transfection. *Bioconjugate Chemistry* **2003**, 14, (4), 782-789.

34. Kim, Y. H.; Gihm, S. H.; Park, C. R.; Lee, K. Y.; Kim, T. W.; Kwon, I. C.; Chung, H.; Jeong, S. Y., Structural characteristics of size-controlled self-aggregates of deoxycholic acid-modified chitosan and their application as a DNA delivery carrier. *Bioconjugate Chemistry* **2001**, 12, (6), 932-8.

35. Yu, H.; Chen, X.; Lu, T.; Sun, J.; Tian, H.; Hu, J.; Wang, Y.; Zhang, P.; Jing, X., Poly(L-lysine)-Graft-Chitosan Copolymers: Synthesis, Characterization, and Gene Transfection Effect. *Biomacromolecules* **2007**, 8, (5), 1425-1435.

36. Wong, K.; Sun, G.; Zhang, X.; Dai, H.; Liu, Y.; He, C.; Leong, K. W., PEI-g-chitosan, a Novel Gene Delivery System with Transfection Efficiency Comparable to Polyethylenimine in Vitro and after Liver Administration in Vivo. *Bioconjugate Chemistry* **2006**, 17, (1), 152-158.
37. Gao, S.; Chen, J.; Xu, X.; Ding, Z.; Yang, Y.-H.; Hua, Z.; Zhang, J., Galactosylated low molecular weight chitosan as DNA carrier for hepatocyte-targeting. *International Journal of Pharmaceutics* **2003**, 255, (1-2), 57-68.
38. Hashimoto, M.; Morimoto, M.; Saimoto, H.; Shigemasa, Y.; Sato, T., Lactosylated chitosan for DNA delivery into hepatocytes: the effect of lactosylation on the physicochemical properties and intracellular trafficking of pDNA/chitosan complexes. *Bioconjugate Chemistry* **2006**, 17, (2), 309-316.
39. Chan, P.; Kurisawa, M.; Chung, J. E.; Yang, Y.-Y., Synthesis and characterization of chitosan-g-poly(ethylene glycol)-folate as a non-viral carrier for tumor-targeted gene delivery. *Biomaterials* **2007**, 28, (3), 540-549.
40. Rigby, P. G., Prolongation of survival of tumour-bearing animals by transfer to "immune" RNA with DEAE-dextran. *Nature* **1969**, 221, (5184), 968-9.
41. Azzam, T.; Eliyahu, H.; Shapira, L.; Linial, M.; Barenholz, Y.; Domb, A. J., Polysaccharide-Oligoamine Based Conjugates for Gene Delivery. *J. Med. Chem.* **2002**, 45, (9), 1817-1824.
42. Azzam, T.; Raskin, A.; Makovitzki, A.; Brem, H.; Vierling, P.; Lineal, M.; Domb, A. J., Cationic Polysaccharides for Gene Delivery. *Macromolecules* **2002**, 35, (27), 9947-9953.
43. Yudovin-Farber, I.; Yanay, C.; Azzam, T.; Linial, M.; Domb, A. J., Quaternary Ammonium Polysaccharides for Gene Delivery. *Bioconjugate Chemistry* **2005**, 16, (5), 1196-1203.

44. Hosseinkhani, H.; Azzam, T.; Tabata, Y.; Domb, A. J., Dextran-spermine polycation: an efficient nonviral vector for in vitro and in vivo gene transfection. *Gene Therapy* **2004**, 11, (2), 194-203.
45. Arima, H.; Hirayama, F.; Okamoto, C. T.; Uekama, K., Recent aspects of cyclodextrin-based pharmaceutical formulations. *Recent Research Developments in Chemical & Pharmaceutical Sciences* **2002**, 2, 155-193.
46. Arima, H.; Kihara, F.; Hirayama, F.; Uekama, K., Enhancement of Gene Expression by Polyamidoamine Dendrimer Conjugates with alpha -, beta -, and gamma -Cyclodextrins. *Bioconjugate Chemistry* **2001**, 12, (4), 476-484.
47. Bellocq, N. C.; Pun, S. H.; Jensen, G. S.; Davis, M. E., Transferrin-Containing, Cyclodextrin Polymer-Based Particles for Tumor-Targeted Gene Delivery. *Bioconjugate Chemistry* **2003**, 14, (6), 1122-1132.
48. Cromwell, W. C.; Bystrom, K.; Eftink, M. R., Cyclodextrin-adamantanecarboxylate inclusion complexes: studies of the variation in cavity size. *J. Phys. Chem.* **1985**, 89, (2), 326-32.
49. Gonzalez, H.; Hwang, S. S. J.; Davis, M. E. Preparation of linear cyclodextrin copolymers. 99-US14298
2000001734, 19990625., 2000.
50. Hwang, S. J.; Bellocq, N. C.; Davis, M. E., Effects of Structure of beta - Cyclodextrin-Containing Polymers on Gene Delivery. *Bioconjugate Chemistry* **2001**, 12, (2), 280-290.
51. Kihara, F.; Arima, H.; Tsutsumi, T.; Hirayama, F.; Uekama, K., In Vitro and In Vivo Gene Transfer by an Optimized alpha -Cyclodextrin Conjugate with Polyamidoamine Dendrimer. *Bioconjugate Chemistry* **2003**, 14, (2), 342-350.

52. Li, J.; Loh, X. J., Cyclodextrin-based supramolecular architectures: Syntheses, structures, and applications for drug and gene delivery. *Advanced Drug Delivery Reviews* **2008**, 60, (9), 1000-1017.
53. Nagai, T.; Ueda, H., Aspects of drug formulation with cyclodextrins. *Compr. Supramol. Chem.* **1996**, 3, 441-450.
54. Pun, S. H.; Bellocq, N. C.; Liu, A.; Jensen, G.; Machemer, T.; Quijano, E.; Schluep, T.; Wen, S.; Engler, H.; Heidel, J.; Davis, M. E., Cyclodextrin-Modified Polyethylenimine Polymers for Gene Delivery. *Bioconjugate Chemistry* **2004**, 15, (4), 831-840.
55. Reineke, T. M.; Davis, M. E., Structural Effects of Carbohydrate-Containing Polycations on Gene Delivery. 2. Charge Center Type. *Bioconjugate Chemistry* **2003**, 14, (1), 255-261.
56. Szejtli, J., Introduction and General Overview of Cyclodextrin Chemistry. *Chem. Rev. (Washington, D. C.)* **1998**, 98, (5), 1743-1753.
57. Popielarski, S. R.; Mishra, S.; Davis, M. E., Structural effects of carbohydrate-containing polycations on gene delivery. 3.cyclodextrin type and functionalization. *Bioconjugate Chemistry* **2003**, 14, (3), 672-678.
58. Pun, S. H.; Davis, M. E., Development of a Nonviral Gene Delivery Vehicle for Systemic Application. *Bioconjugate Chemistry* **2002**, 13, (3), 630-639.
59. Reineke, T. M.; Davis, M. E., Structural Effects of Carbohydrate-Containing Polycations on Gene Delivery. 1. Carbohydrate Size and Its Distance from Charge Centers. *Bioconjugate Chemistry* **2003**, 14, (1), 247-254.
60. Kulkarni, R. P.; Mishra, S.; Fraser, S. E.; Davis, M. E., Single cell kinetics of intracellular, nonviral, nucleic acid delivery vehicle acidification and trafficking. *Bioconjugate Chemistry* **2005**, 16, (4), 986-994.

61. Ahn, C.-H.; Chae, S. Y.; Bae, Y. H.; Kim, S. W., Biodegradable poly(ethylenimine) for plasmid DNA delivery. *Journal of Controlled Release* **2002**, 80, (1-3), 273-282.
62. Ahn, H. H.; Lee, J. H.; Kim, K. S.; Lee, J. Y.; Kim, M. S.; Khang, G.; Lee, I. W.; Lee, H. B., Polyethyleneimine-mediated gene delivery into human adipose derived stem cells. *Biomaterials* **2008**, 29, (15), 2415-2422.
63. Arote, R.; Kim, T.-H.; Kim, Y.-K.; Hwang, S.-K.; Jiang, H.-L.; Song, H.-H.; Nah, J.-W.; Cho, M.-H.; Cho, C.-S., A biodegradable poly(ester amine) based on polycaprolactone and polyethylenimine as a gene carrier. *Biomaterials* **2006**, 28, (4), 735-744.
64. Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P., A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* **1995**, 92, (16), 7297-301.
65. Zhang, M.; Liu, M.; Xue, Y.-N.; Huang, S.-W.; Zhuo, R.-X., Polyaspartamide-Based Oligo-ethylenimine Brushes with High Buffer Capacity and Low Cytotoxicity for Highly Efficient Gene Delivery. *Bioconjugate Chemistry* **2009**, 20, (3), 440-446.
66. Xiong, M. P.; Forrest, M. L.; Karls, A. L.; Kwon, G. S., Biotin-Triggered Release of Poly(ethylene glycol)-Avidin from Biotinylated Polyethylenimine Enhances in Vitro Gene Expression. *Bioconjugate Chemistry* **2007**, 18, (3), 746-753.
67. Suh, J.; Paik, H.-J.; Hwang, B. K., Ionization of poly(ethylenimine) and poly(allylamine) at various pH's. *Bioorg. Chem.* **1994**, 22, (3), 318-27.
68. Sung, S.-J.; Min, S. H.; Cho, K. Y.; Lee, S.; Min, Y.-J.; Yeom, Y., II; Park, J.-K., Effect of polyethylene glycol on gene delivery of polyethylenimine. *Biol. Pharm. Bull.* **2003**, 26, (4), 492-500.

69. Thomas, M.; Klibanov, A. M., Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **2002**, 99, (23), 14640-14645.
70. Vinogradov, S.; Batrakova, E.; Kabanov, A., Poly(ethylene glycol)-polyethylenimine NanoGel particles: novel drug delivery systems for antisense oligonucleotides. *Colloids Surf., B* **1999**, 16, (1-4), 291-304.
71. Wang, D.; Narang, A. S.; Kotb, M.; Gaber, A. O.; Miller, D. D.; Kim, S. W.; Mahato, R. I., Novel Branched Poly(Ethylenimine)-Cholesterol Water-Soluble Lipopolymers for Gene Delivery. *Biomacromolecules* **2002**, 3, (6), 1197-1207.
72. Petersen, H.; Merdan, T.; Kunath, K.; Fischer, D.; Kissel, T., Poly(ethylenimine-co-L-lactamide-co-succinamide): A Biodegradable Polyethylenimine Derivative with an Advantageous pH-Dependent Hydrolytic Degradation for Gene Delivery. *Bioconjugate Chemistry* **2002**, 13, (4), 812-821.
73. Suh, W.; Han, S.-O.; Yu, L.; Kim, S. W., An angiogenic, endothelial-cell-targeted polymeric gene carrier. *Molecular Therapy* **2002**, 6, (5), 664-672.
74. Park, M. R.; Han, K. O.; Han, I. K.; Cho, M. H.; Nah, J. W.; Choi, Y. J.; Cho, C. S., Degradable polyethylenimine-alt-poly(ethylene glycol) copolymers as novel gene carriers. *Journal of Controlled Release* **2005**, 105, (3), 367-380.
75. Behr, J. P., The proton sponge. A trick to enter cells the viruses did not exploit. *Chimia* **1997**, 51, (1/2), 34-36.
76. Godbey, W. T.; Wu, K. K.; Hirasaki, G. J.; Mikos, A. G., Improved packing of poly(ethylenimine)/DNA complexes increases transfection efficiency. *Gene Therapy* **1999**, 6, (8), 1380-1388.
77. Godbey, W. T.; Wu, K. K.; Mikos, A. G., Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proceedings of the National Academy of Sciences of the United States of America* **1999**, 96, (9), 5177-5181.

78. Godbey, W. T.; Wu, K. K.; Mikos, A. G., Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. *J Biomed Mater Res* **1999**, 45, (3), 268-75.
79. Dunlap, D. D.; Maggi, A.; Soria, M. R.; Monaco, L., Nanoscopic structure of DNA condensed for gene delivery. *Nucleic Acids Res.* **1997**, 25, (15), 3095-101.
80. Banerjee, P.; Weissleder, R.; Bogdanov, A., Jr., Linear Polyethyleneimine Grafted to a Hyperbranched Poly(ethylene glycol)-like Core: A Copolymer for Gene Delivery. *Bioconjugate Chemistry* **2006**, 17, (1), 125-131.
81. Brus, C.; Petersen, H.; Aigner, A.; Czubayko, F.; Kissel, T., Physicochemical and Biological Characterization of Polyethylenimine-graft-Poly(ethylene glycol) Block Copolymers as a Delivery System for Oligonucleotides and Ribozymes. *Bioconjugate Chemistry* **2004**, 15, (4), 677-684.
82. Burke, R. S.; Pun, S. H., Extracellular Barriers to in Vivo PEI and PEGylated PEI Polyplex-Mediated Gene Delivery to the Liver. *Bioconjugate Chemistry* **2008**, 19, (3), 693-704.
83. Chemin, I.; Moradpour, D.; Wieland, S.; Offensperger, W. B.; Walter, E.; Behr, J. P.; Blum, H. E., Liver-directed gene transfer: a linear polyethylenimine derivative mediates highly efficient DNA delivery to primary hepatocytes in vitro and in vivo. *Journal of viral hepatitis* **1998**, 5, (6), 369-75.
84. Cheng, H.; Zhu, J.-L.; Zeng, X.; Jing, Y.; Zhang, X.-Z.; Zhuo, R.-X., Targeted Gene Delivery Mediated by Folate-polyethylenimine-block-poly(ethylene glycol) with Receptor Selectivity. *Bioconjugate Chemistry* **2009**, 20, (3), 481-487.
85. Diebold, S. S.; Kursu, M.; Wagner, E.; Cotten, M.; Zenke, M., Mannose polyethylenimine conjugates for targeted DNA delivery into dendritic cells. *Journal of Biological Chemistry* **1999**, 274, (27), 19087-19094.

86. Petersen, H.; Fechner, P. M.; Martin, A. L.; Kunath, K.; Stolnik, S.; Roberts, C. J.; Fischer, D.; Davies, M. C.; Kissel, T., Polyethylenimine-graft-Poly(ethylene glycol) Copolymers: Influence of Copolymer Block Structure on DNA Complexation and Biological Activities as Gene Delivery System. *Bioconjugate Chemistry* **2002**, 13, (4), 845-854.
87. Ogris, M.; Brunner, S.; Schuller, S.; Kircheis, R.; Wagner, E., PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Therapy* **1999**, 6, (4), 595-605.
88. Rudolph, C.; Schillinger, U.; Plank, C.; Gessner, A.; Nicklaus, P.; Muller, R.; Rosenecker, J., Nonviral gene delivery to the lung with copolymer-protected and transferrin-modified polyethylenimine. *Biochim Biophys Acta* **2002**, 1573, (1), 75-83.
89. Shuai, X.; Merdan, T.; Unger, F.; Wittmar, M.; Kissel, T., Novel Biodegradable Ternary Copolymers hy-PEI-g-PCL-b-PEG: Synthesis, Characterization, and Potential as Efficient Nonviral Gene Delivery Vectors. *Macromolecules* **2003**, 36, (15), 5751-5759.
90. Cho, K. C.; Kim, S. H.; Jeong, J. H.; Park, T. G., Folate receptor-mediated gene delivery using folate-poly(ethylene glycol)-poly(L-lysine) conjugate. *Macromol. Biosci.* **2005**, 5, (6), 512-519.
91. Suk, J. S.; Suh, J.; Choy, K.; Lai, S. K.; Fu, J.; Hanes, J., Gene delivery to differentiated neurotypic cells with RGD and HIV Tat peptide functionalized polymeric nanoparticles. *Biomaterials* **2006**, 27, (29), 5143-5150.
92. Kim, W. J.; Yockman, J. W.; Lee, M.; Jeong, J. H.; Kim, Y.-H.; Kim, S. W., Soluble Flt-1 gene delivery using PEI-g-PEG-RGD conjugate for anti-angiogenesis. *Journal of Controlled Release* **2005**, 106, (1-2), 224-234.

93. Kunath, K.; Merdan, T.; Hegener, O.; Haeberlein, H.; Kissel, T., Integrin targeting using RGD-PEI conjugates for in vitro gene transfer. *J. Gene Med.* **2003**, 5, (7), 588-599.
94. Ogris, M.; Steinlein, P.; Kursa, M.; Mechtler, K.; Kircheis, R.; Wagner, E., The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Therapy* **1998**, 5, (10), 1425-1433.
95. Gosselin, M. A.; Guo, W.; Lee, R. J., Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. *Bioconjugate Chemistry* **2001**, 12, (6), 989-94.
96. Shuai, X.; Merdan, T.; Unger, F.; Kissel, T., Supramolecular Gene Delivery Vectors Showing Enhanced Transgene Expression and Good Biocompatibility. *Bioconjugate Chemistry* **2005**, 16, (2), 322-329.
97. Forrest, M. L.; Koerber, J. T.; Pack, D. W., A Degradable Polyethylenimine Derivative with Low Toxicity for Highly Efficient Gene Delivery. *Bioconjugate Chemistry* **2003**, 14, (5), 934-940.
98. Kim, T.-I.; Seo, H. J.; Choi, J. S.; Yoon, J. K.; Baek, J.; Kim, K.; Park, J.-S., Synthesis of biodegradable cross-Linked Poly(beta -amino ester) for gene delivery and its modification, inducing enhanced transfection efficiency and stepwise Degradation. *Bioconjugate Chemistry* **2005**, 16, (5), 1140-1148.
99. Cherng, J. Y.; van de Wetering, P.; Talsma, H.; Crommelin, D. J.; Hennink, W. E., Effect of size and serum proteins on transfection efficiency of poly ((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles. *Pharmaceutical Research* **1996**, 13, (7), 1038-42.
100. van de Wetering, P.; Cherng, J.-Y.; Talsma, H.; Hennink, W. E., Relation between transfection efficiency and cytotoxicity of poly[2-(dimethylamino)ethyl methacrylate]/plasmid complexes. *Journal of Controlled Release* **1997**, 49, (1), 59-69.

101. Van de Wetering, P.; Moret, E. E.; Schuurmans-Nieuwenbroek, N. M. E.; Van Steenberghe, M. J.; Hennink, W. E., Structure-Activity Relationships of Water-Soluble Cationic Methacrylate/Methacrylamide Polymers for Nonviral Gene Delivery. *Bioconjugate Chemistry* **1999**, 10, (4), 589-597.
102. Funhoff, A. M.; van Nostrum, C. F.; Koning, G. A.; Schuurmans-Nieuwenbroek, N. M. E.; Crommelin, D. J. A.; Hennink, W. E., Endosomal Escape of Polymeric Gene Delivery Complexes Is Not Always Enhanced by Polymers Buffering at Low pH. *Biomacromolecules* **2004**, 5, (1), 32-39.
103. Dubruel, P.; Christiaens, B.; Vanloo, B.; Bracke, K.; Rosseneu, M.; Vandekerckhove, J.; Schacht, E., Physicochemical and biological evaluation of cationic polymethacrylates as vectors for gene delivery. *Eur. J. Pharm. Sci.* **2003**, 18, (3-4), 211-220.
104. Funhoff, A. M.; Van Nostrum, C. F.; Lok, M. C.; Fretz, M. M.; Crommelin, D. J. A.; Hennink, W. E., Poly(3-guanidinopropyl methacrylate): A Novel Cationic Polymer for Gene Delivery. *Bioconjugate Chemistry* **2004**, 15, (6), 1212-1220.
105. van de Wetering, P.; Cherng, J. Y.; Talsma, H.; Crommelin, D. J. A.; Hennink, W. E., 2-(dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents. *Journal of Controlled Release* **1998**, 53, (1-3), 145-153.
106. van de Wetering, P.; Schuurmans-Nieuwenbroek, N. M. E.; van Steenberghe, M. J.; Crommelin, D. J. A.; Hennink, W. E., Copolymers of 2-(dimethylamino)ethyl methacrylate with ethoxytriethylene glycol methacrylate or N-vinylpyrrolidone as gene transfer agents. *Journal of Controlled Release* **2000**, 64, (1-3), 193-203.
107. Mastrobattista, E.; Kapel, R. H.; Eggenhuisen, M. H.; Roholl, P. J.; Crommelin, D. J.; Hennink, W. E.; Storm, G., Lipid-coated polyplexes for targeted gene delivery to ovarian carcinoma cells. *Cancer Gene Ther.* **2001**, 8, (6), 405-13.

108. van Steenis, J. H.; van Maarseveen, E. M.; Verbaan, F. J.; Verrijk, R.; Crommelin, D. J. A.; Storm, G.; Hennink, W. E., Preparation and characterization of folate-targeted pEG-coated pDMAEMA-based polyplexes. *Journal of controlled release official journal of the Controlled Release Society* **2003**, 87, (1-3), 167-76.
109. Lim, D. W.; Yeom, Y. I.; Park, T. G., Poly(DMAEMA-NVP)-b-PEG-galactose as gene delivery vector for hepatocytes. *Bioconjugate Chemistry* **2000**, 11, (5), 688-95.
110. Wakebayashi, D.; Nishiyama, N.; Yamasaki, Y.; Itaka, K.; Kanayama, N.; Harada, A.; Nagasaki, Y.; Kataoka, K., Lactose-conjugated polyion complex micelles incorporating plasmid DNA as a targetable gene vector system: their preparation and gene transfecting efficiency against cultured HepG2 cells. *Journal of Controlled Release* **2004**, 95, (3), 653-664.
111. Oishi, M.; Kataoka, K.; Nagasaki, Y., pH-Responsive Three-Layered PEGylated Polyplex Micelle Based on a Lactosylated ABC Triblock Copolymer as a Targetable and Endosome-Disruptive Nonviral Gene Vector. *Bioconjugate Chemistry* **2006**, 17, (3), 677-688.
112. Funhoff, A. M.; van Nostrum, C. F.; Janssen, A. P. C. A.; Fens, M. H. A. M.; Crommelin, D. J. A.; Hennink, W. E., Polymer side-chain degradation as a tool to control the destabilization of polyplexes. *Pharmaceutical Research* **2004**, 21, (1), 170-176.
113. Luten, J.; Akeroyd, N.; Funhoff, A.; Lok, M. C.; Talsma, H.; Hennink, W. E., Methacrylamide Polymers with Hydrolysis-Sensitive Cationic Side Groups as Degradable Gene Carriers. *Bioconjugate Chemistry* **2006**, 17, (4), 1077-1084.
114. Jiang, X.; Lok, M. C.; Hennink, W. E., Degradable-brushed poly(hydroxyethylmethacrylate)-poly(2-(dimethylamino)ethyl methacrylate)

synthesized via ATRP and click chemistry for gene delivery. *Bioconjugate Chemistry* **2007**, 18, (6), 2077-2084.

115. Fuller, W. D.; Verlander, M. S.; Goodman, M., A procedure for the facile synthesis of amino-acid N-carboxyanhydrides. *Biopolymers* **1976**, 15, (9), 1869-71.

116. Laemmli, U. K., Characterization of DNA condensates induced by poly(ethylene oxide) and polylysine. *Proceedings of the National Academy of Sciences of the United States of America* **1975**, 72, (11), 4288-92.

117. Ahn, C.-H.; Chae, S. Y.; Bae, Y. H.; Kim, S. W., Synthesis of biodegradable multi-block copolymers of poly(L-lysine) and poly(ethylene glycol) as a non-viral gene carrier. *Journal of Controlled Release* **2004**, 97, (3), 567-574.

118. Bennis, J. M.; Choi, J. S.; Mahato, R. I.; Park, J. S.; Kim, S. W., pH-sensitive cationic polymer gene delivery vehicle: N-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer. *Bioconjugate Chemistry* **2000**, 11, (5), 637-45.

119. Choi, Y. H.; Liu, F.; Park, J. S.; Kim, S. W., Lactose-poly(ethylene glycol)-grafted poly-L-lysine as hepatoma cell-targeted gene carrier. *Bioconjugate Chemistry* **1998**, 9, (6), 708-18.

120. Han, J.; Il Yeom, Y., Specific gene transfer mediated by galactosylated poly-L-lysine into hepatoma cells. *International Journal of Pharmaceutics* **2000**, 202, (1-2), 151-60.

121. Katayose, S.; Kataoka, K., Water-Soluble Polyion Complex Associates of DNA and Poly(ethylene glycol)-Poly(L-lysine) Block Copolymer. *Bioconjugate Chemistry* **1997**, 8, (5), 702-707.

122. Kwok, D. Y.; Coffin, C. C.; Lollo, C. P.; Jovenal, J.; Banaszczyk, M. G.; Mullen, P.; Phillips, A.; Amini, A.; Fabrycki, J.; Bartholomew, R. M.; Brostoff, S. W.; Carlo, D. J., Stabilization of poly-L-lysine/DNA polyplexes for in vivo gene delivery to the liver. *Biochim Biophys Acta* **1999**, 1444, (2), 171-90.

123. Mannisto, M.; Vanderkerken, S.; Toncheva, V.; Elomaa, M.; Ruponen, M.; Schacht, E.; Urtti, A., Structure-activity relationships of poly(L-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. *Journal of Controlled Release* **2002**, 83, (1), 169-182.
124. Trubetskoy, V. S.; Torchilin, V. P.; Kennel, S. J.; Huang, L., Use of N-terminal modified poly(L-lysine)-antibody conjugate as a carrier for targeted gene delivery in mouse lung endothelial cells. *Bioconjugate Chemistry* **1992**, 3, (4), 323-7.
125. Wolfert, M. A.; Seymour, L. W., Chloroquine and amphipathic peptide helices show synergistic transfection in vitro. *Gene Therapy* **1998**, 5, (3), 409-14.
126. Doody, A. M.; Korley, J. N.; Dang, K. P.; Zawaneh, P. N.; Putnam, D., Characterizing the structure/function parameter space of hydrocarbon-conjugated branched polyethylenimine for DNA delivery in vitro. *Journal of Controlled Release* **2006**, 116, (2), 227-237.
127. Lynn, D. M.; Anderson, D. G.; Putnam, D.; Langer, R., Accelerated discovery of synthetic transfection vectors: Parallel synthesis and screening of degradable polymer library. *Journal of the American Chemical Society* **2001**, 123, (33), 8155-8156.
128. Putnam, D., Polymers for gene delivery across length scales. *Nature Materials* **2006**, 5, (6), 439-451.
129. Putnam, D.; Gentry, C. A.; Pack, D. W.; Langer, R., Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, 98, (3), 1200-+.
130. Putnam, D.; Langer, R., Poly(4-hydroxy-L-proline ester): Low-Temperature Polycondensation and Plasmid DNA Complexation. *Macromolecules* **1999**, 32, (11), 3658-3662.

131. Ohsaki, M.; Okuda, T.; Wada, A.; Hirayama, T.; Niidome, T.; Aoyagi, H., In Vitro Gene Transfection Using Dendritic Poly(L-lysine). *Bioconjugate Chemistry* **2002**, 13, (3), 510-517.
132. Liu, G.; Molas, M.; Grossmann, G. A.; Pasumarth, M.; Perales, J. C.; Cooper, M. J.; Hanson, R. W., Biological properties of poly-L-lysine-DNA complexes generated by cooperative binding of the polycation. *The Journal of biological chemistry* **2001**, 276, (37), 34379-87.
133. Harada, A.; Togawa, H.; Kataoka, K., Physicochemical properties and nuclease resistance of antisense-oligodeoxynucleotides entrapped in the core of polyion complex micelles composed of poly(ethylene glycol)-poly(L-lysine) block copolymers. *Eur J Pharm Sci* **2001**, 13, (1), 35-42.
134. Wolfert, M. A.; Schacht, E. H.; Toncheva, V.; Ulbrich, K.; Nazarova, O.; Seymour, L. W., Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers. *Human Gene Therapy* **1996**, 7, (17), 2123-2133.
135. Bikram, M.; Ahn, C.-H.; Chae, S. Y.; Lee, M.; Yockman, J. W.; Kim, S. W., Biodegradable Poly(ethylene glycol)-co-poly(L-lysine)-g-histidine Multiblock Copolymers for Nonviral Gene Delivery. *Macromolecules* **2004**, 37, (5), 1903-1916.
136. Park, S.; Healy, K. E., Compositional regulation of poly(lysine-g-(lactide-b-ethylene glycol))-DNA complexation and stability. *Journal of Controlled Release* **2004**, 95, (3), 639-651.
137. Park, S.; Healy, K. E., Nanoparticulate DNA Packaging Using Terpolymers of Poly(lysine-g-(lactide-b-ethylene glycol)). *Bioconjugate Chemistry* **2003**, 14, (2), 311-319.

138. Mousazadeh, M.; Palizban, A.; Salehi, R.; Salehi, M., Gene delivery to brain cells with apoprotein E derived peptide conjugated to polylysine (apoEdp-PLL). *Journal of Drug Targeting* **2007**, 15, (3), 226-230.
139. Plank, C.; Zatloukal, K.; Cotten, M.; Mechtler, K.; Wagner, E., Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconjugate Chemistry* **1992**, 3, (6), 533-9.
140. Wagner, E.; Cotten, M.; Foisner, R.; Birnstiel, M. L., Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proceedings of the National Academy of Sciences of the United States of America* **1991**, 88, (10), 4255-9.
141. Wagner, E.; Zenke, M.; Cotten, M.; Beug, H.; Birnstiel, M. L., Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proceedings of the National Academy of Sciences of the United States of America* **1990**, 87, (9), 3410-4.
142. Kiely, D. E.; Chen, L.; Lin, T. H., Hydroxylated nylons based on unprotected esterified D-glucaric acid by simple condensation reactions. *Journal of the American Chemical Society* **1994**, 116, (2), 571-8.
143. Liu, Y.; Wenning, L.; Lynch, M.; Reineke, T. M., New Poly(D-glucaramidoamine)s Induce DNA Nanoparticle Formation and Efficient Gene Delivery into Mammalian Cells. *Journal of the American Chemical Society* **2004**, 126, (24), 7422-7423.
144. Liu, Y.; Reineke, T. M., Hydroxyl Stereochemistry and Amine Number within Poly(glycoamidoamine)s Affect Intracellular DNA Delivery. *Journal of the American Chemical Society* **2005**, 127, (9), 3004-3015.

145. Liu, Y.; Reineke, T. M., Poly(glycoamidoamine)s for Gene Delivery: Stability of Polyplexes and Efficacy with Cardiomyoblast Cells. *Bioconjugate Chemistry* **2006**, 17, (1), 101-108.
146. Reineke, T. M.; Liu, Y., Synthesis and characterization of polyhydroxylamides for DNA delivery. *PMSE Preprints* **2003**, 89, 53-54.
147. Liu, Y.; Reineke, T. M., Poly(glycoamidoamine)s for Gene Delivery. Structural Effects on Cellular Internalization, Buffering Capacity, and Gene Expression. *Bioconjugate Chemistry* **2007**, 18, (1), 19-30.
148. Lee, C.-C.; Liu, Y.; Reineke, T. M., General Structure-Activity Relationship for Poly(glycoamidoamine)s: The Effect of Amine Density on Cytotoxicity and DNA Delivery Efficiency. *Bioconjugate Chemistry* **2008**, 19, (2), 428-440.
149. Ferruti, P.; Marchisio, M. A.; Barbucci, R., Synthesis, physicochemical properties and biomedical applications of poly(amido-amine)s. *Polymer* **1985**, 26, (9), 1336-48.
150. Barbucci, R.; Casolaro, M.; Ferruti, P.; Barone, V.; Leli, F.; Oliva, L., Macroinorganics. 7. Property structure relationships for polymeric bases whose monomeric units behave independently toward protonation. *Macromolecules* **1981**, 14, (5), 1203-9.
151. Richardson, S.; Ferruti, P.; Duncan, R., Poly(amidoamine)s as potential endosomolytic polymers: evaluation in vitro and body distribution in normal and tumour-bearing animals. *Journal of Drug Targeting* **1999**, 6, (6), 391-404.
152. Ferruti, P.; Manzoni, S.; Richardson, S. C. W.; Duncan, R.; Patrick, N. G.; Mendichi, R.; Casolaro, M., Amphoteric Linear Poly(amido-amine)s as Endosomolytic Polymers: Correlation between Physicochemical and Biological Properties. *Macromolecules* **2000**, 33, (21), 7793-7800.

153. Richardson, S. C.; Patrick, N. G.; Man, Y. K.; Ferruti, P.; Duncan, R., Poly(amidoamine)s as potential nonviral vectors: ability to form interpolyelectrolyte complexes and to mediate transfection in vitro. *Biomacromolecules* **2001**, 2, (3), 1023-8.
154. Franchini, J.; Ranucci, E.; Ferruti, P.; Rossi, M.; Cavalli, R., Synthesis, Physicochemical Properties, and Preliminary Biological Characterizations of a Novel Amphoteric Agmatine-Based Poly(amidoamine) with RGD-Like Repeating Units. *Biomacromolecules* **2006**, 7, (4), 1215-1222.
155. Ferruti, P.; Franchini, J.; Bencini, M.; Ranucci, E.; Zara, G. P.; Serpe, L.; Primo, L.; Cavalli, R., Prevailingly Cationic Agmatine-Based Amphoteric Polyamidoamine as a Nontoxic, Nonhemolytic, and "Stealthlike" DNA Complexing Agent and Transfection Promoter. *Biomacromolecules* **2007**, 8, (5), 1498-1504.
156. Lin, C.; Zhong, Z.; Lok, M. C.; Jiang, X.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J., Linear poly(amido amine)s with secondary and tertiary amino groups and variable amounts of disulfide linkages: Synthesis and in vitro gene transfer properties. *Journal of Controlled Release* **2006**, 116, (2), 130-137.
157. Lin, C.; Zhong, Z.; Lok, M. C.; Jiang, X.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J., Novel Bio reducible Poly(amido amine)s for Highly Efficient Gene Delivery. *Bioconjugate Chemistry* **2007**, 18, (1), 138-145.
158. Lin, C.; Zhong, Z.; Lok, M. C.; Jiang, X.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J., Random and block copolymers of bio reducible poly(amido amine)s with high- and low-basicity amino groups: Study of DNA condensation and buffer capacity on gene transfection. *Journal of Controlled Release* **2007**, 123, (1), 67-75.

159. Lim, Y.-b.; Choi, Y. H.; Park, J.-s., A Self-Destroying Polycationic Polymer: Biodegradable Poly(4-hydroxy-L-proline ester). *Journal of the American Chemical Society* **1999**, 121, (24), 5633-5639.
160. Kwon, H. Y.; Langer, R., Pseudopoly(amino acids): a study of the synthesis and characterization of poly(trans-4-hydroxy-N-acyl-L-proline esters). *Macromolecules* **1989**, 22, (8), 3250-5.
161. Li, Z.; Huang, L., Sustained delivery and expression of plasmid DNA based on biodegradable polyester, poly(D, L-lactide-co-4-hydroxy-L-proline). *Journal of Controlled Release* **2004**, 98, (3), 437-446.
162. Lim, Y.-b.; Kim, C.-h.; Kim, K.; Kim, S. W.; Park, J.-s., Development of a safe gene delivery system using biodegradable polymer, poly[alpha -(4-aminobutyl)-L-glycolic acid]. *Journal of the American Chemical Society* **2000**, 122, (27), 6524-6525.
163. Lim, Y.-B.; Han, S.-O.; Kong, H.-U.; Lee, Y.; Park, J.-S.; Jeong, B.; Kim, S. W., Biodegradable polyester, poly[alpha -(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier. *Pharmaceutical Research* **2000**, 17, (7), 811-816.
164. Koh, J. J.; Ko, K. S.; Lee, M.; Han, S.; Park, J. S.; Kim, S. W., Degradable polymeric carrier for the delivery of IL-10 plasmid DNA to prevent autoimmune insulinitis of NOD mice. *Gene Therapy* **2000**, 7, (24), 2099-104.
165. Ko, K. S.; Lee, M.; Koh, J. J.; Kim, S. W., Combined administration of plasmids encoding IL-4 and IL-10 prevents the development of autoimmune diabetes in nonobese diabetic mice. *Mol Ther* **2001**, 4, (4), 313-6.
166. Lee, J. H.; Lim, Y.-b.; Choi, J. S.; Lee, Y.; Kim, T.-i.; Kim, H. J.; Yoon, J. K.; Kim, K.; Park, J.-s., Polyplexes Assembled with Internally Quaternized PAMAM-OH Dendrimer and Plasmid DNA Have a Neutral Surface and Gene Delivery Potency. *Bioconjugate Chemistry* **2003**, 14, (6), 1214-1221.

167. Lynn, D. M.; Langer, R., Degradable poly(beta-amino esters): Synthesis, characterization, and self-assembly with plasmid DNA. *Journal of the American Chemical Society* **2000**, 122, (44), 10761-10768.
168. Lynn, D. M.; Anderson, D. G.; Putnam, D.; Langer, R., Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of a degradable polymer library. *Journal of the American Chemical Society* **2001**, 123, (33), 8155-6.
169. Green, J. J.; Zugates, G. T.; Tedford, N. C.; Huang, Y.-H.; Griffith, L. G.; Lauffenburger, D. A.; Sawicki, J. A.; Langer, R.; Anderson, D. G., Combinatorial modification of degradable polymers enables transfection of human cells comparable to adenovirus. *Advanced Materials (Weinheim, Germany)* **2007**, 19, (19), 2836-2842.
170. Anderson, D. G.; Lynn, D. M.; Langer, R., Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery. *Angewandte Chemie, International Edition* **2003**, 42, (27), 3153-3158.
171. Zugates, G. T.; Tedford, N. C.; Zumbuehl, A.; Jhunjhunwala, S.; Kang, C. S.; Griffith, L. G.; Lauffenburger, D. A.; Langer, R.; Anderson, D. G., Gene Delivery Properties of End-Modified Poly(beta -amino ester)s. *Bioconjugate Chemistry* **2007**, 18, (6), 1887-1896.
172. Wu, D.; Liu, Y.; Jiang, X.; Chen, L.; He, C.; Goh, S. H.; Leong, K. W., Evaluation of hyperbranched poly(amino ester)s of amine constitutions similar to polyethylenimine for DNA delivery. *Biomacromolecules* **2005**, 6, (6), 3166-3173.
173. Wu, D.; Liu, Y.; Jiang, X.; He, C.; Goh, S. H.; Leong, K. W., Hyperbranched Poly(amino ester)s with Different Terminal Amine Groups for DNA Delivery. *Biomacromolecules* **2006**, 7, (6), 1879-1883.
174. Zhong, Z.; Song, Y.; Engbersen, J. F. J.; Lok, M. C.; Hennink, W. E.; Feijen, J., A versatile family of degradable non-viral gene carriers based on hyperbranched poly(ester amine)s. *Journal of Controlled Release* **2005**, 109, (1-3), 317-329.

175. Mujumdar, A. N.; Young, S. G.; Merker, R. L.; Magill, J. H., A study of solution polymerization of polyphosphazenes. *Macromolecules* **1990**, 23, (1), 14-21.
176. Allcock, H. R.; McIntosh, M. B.; Klingenberg, E. H.; Napierala, M. E., Functionalized Polyphosphazenes: Polymers with Pendent Tertiary Trialkylamino Groups. *Macromolecules* **1998**, 31, (16), 5255-5263.
177. Luten, J.; van Steenis, J. H.; van Someren, R.; Kemmink, J.; Schuurmans-Nieuwenbroek, N. M. E.; Koning, G. A.; Crommelin, D. J. A.; van Nostrum, C. F.; Hennink, W. E., Water-soluble biodegradable cationic polyphosphazenes for gene delivery. *Journal of controlled release official journal of the Controlled Release Society* **2003**, 89, (3), 483-97.
178. Penczek, S.; Pretula, J., High-molecular-weight poly (alkylene phosphates) and preparation of amphiphilic polymers thereof. *Macromolecules* **1993**, 26, (9), 2228-33.
179. Wang, J.; Mao, H.-Q.; Leong, K. W., A Novel Biodegradable Gene Carrier Based on Polyphosphoester. *Journal of the American Chemical Society* **2001**, 123, (38), 9480-9481.
180. Wang, J.; Huang, S.-W.; Zhang, P.-C.; Mao, H.-Q.; Leong, K. W., Effect of side-chain structures on gene transfer efficiency of biodegradable cationic polyphosphoesters. *International Journal of Pharmaceutics* **2003**, 265, (1-2), 75-84.
181. Wang, J.; Zhang, P.-C.; Lu, H.-F.; Ma, N.; Wang, S.; Mao, H.-Q.; Leong, K. W., New polyphosphoramidate with a spermidine side chain as a gene carrier. *Journal of Controlled Release* **2002**, 83, (1), 157-168.

CHAPTER 2
SYNTHESIS AND CHARACTERIZATION OF IONIC CHARGED WATER
SOLUBLE ARGININE BASED POLY (ESTER AMIDE)

Jun Wu¹, Martha A. Mutschler², Chih-Chang Chu^{1,3}

¹ Department of Biomedical Engineering, Cornell University, Ithaca, NY 14853-4401,
USA

² Department of Plant Breeding, Cornell University, Ithaca, NY, 14853-4401, USA

³ Department of Fiber Science and Apparel Design, Cornell University, Ithaca, NY
14853-4401, USA

2.A Abstract

A family of water soluble and positively charged L-arginine based poly (ester amide)s (Arg-PEAs) was synthesized and characterized. These biodegradable polymers consist of 3 nontoxic building blocks: L-arginine, diols, and dicarboxylic acids. The Arg-PEAs were prepared by solution polycondensation reaction of tetra-*p*-toluenesulfonic acids salts of bis-(L-arginine) α , ω -alkylene diesters and di-*p*-nitrophenyl esters of dicarboxylic acids. Optimal conditions of the monomers and polymers synthesis were investigated, and the monomers and Arg-PEAs were chemically characterized. Arg-PEAs were found to have good solubility in water and many other polar solvents. Structure-function relationship of the Arg-PEAs revealed that changing the number of methylene groups in the diol or/and diacid segment could finely tune the hydrophobic and cationic properties of the Arg-PEAs. MTT assay showed that all the prepared Arg-PEAs were non-toxic to the cell lines even at very large doses. Arg-PEAs with double bond functionality could be photo-crosslinked with polyethylene glycol diacrylate (PEGDA) to form cationic hybrid hydrogels.

2.B Introduction

In recent years, due to the fast growing of biotechnology and pharmaceutical science, many new biodegradable and biocompatible synthetic polymers have been developed¹⁻⁹. For examples, absorbable aliphatic polyesters, such as polylactide (PLA), polyglycolide (PGA), poly- ϵ -caprolactone (PCL) and their copolymers are the most well-known and widely used polymeric biomaterials because of their good biocompatibility, biodegradability, mechanical and processing property^{1, 2, 5, 6, 9-12}. These FDA-approved absorbable aliphatic polyesters have been widely used in wound

closure, tissue engineering, drug delivery and medical implants^{1, 2, 5, 6, 9-12}. However, all of these aliphatic polyester-based synthetic absorbable biomaterials are soluble in organic solvents only. Their poor water solubility greatly limits their applications in an aqueous-based biological system.

The rapid development of biotechnology demands new generations of water soluble, biocompatible and biodegradable synthetic polymers with various physicochemical, functional and biological properties. For this purpose, recently, many kinds of chemical modifications have been applied to the current available synthetic biodegradable and biocompatible polymers to meet the demands in biomedical applications. One approach is to introduce the polyether into the absorbable polymers' main or side chain, such as the PLA-*b*-PEG¹³ and PLL-*b*-PEG^{14, 15}, which have been widely investigated in the areas of antibiofouling, self-assembly, drug/gene delivery and nanotechnology. The incorporation of polyether segment will improve the solubility, hydrophilicity of absorbable aliphatic polyesters and increase the circulation time in vivo. Another interesting approach is to introduce the poly (amino acid)s or polypeptide into the polymer backbone. The introduction of natural amino acids would bring the polymer many new properties, such as functionality, biodegradability and charge property in addition to the improvement of hydrophilicity. One example for this approach is polyester-*b*-poly (amino acid)s, such as PLA-co-PLL and PCL-co-PLL, which have been widely used in the drug delivery and tissue engineering scaffold area^{4, 16, 17}. However, these modifications could hardly make the water insoluble absorbable aliphatic polyesters into water soluble polymers.

In this study, we report the design of a water soluble, biocompatible and biodegradable polymer family for biotechnology applications. This polymer family

belongs to the amino acid-based poly (ester amide) (PEA, Figure 2.1) category. Amino acid based-PEAs are biodegradable and biocompatible synthetic polymers having both ester and amide linkages on their backbones, which bring good mechanical and biological properties with enzyme-catalyzed biodegradability^{5, 8, 18-22}. Combining the favorable properties of polyesters, polyamides, and poly (amino acid)s, amino acid-based PEAs have shown very promising applications in the biomedical and biotechnology area^{5, 8, 18-22}. The PEA backbone consists of nontoxic building blocks like α -amino acids, fatty diols and aliphatic dicarboxylic acids. The varieties of combinations of the 3 types of building blocks offer a variety of PEAs for different purposes. And functional groups, such as double bonds, amine or hydroxyl groups, could be incorporated into the polymer chains^{8, 20}. Due to the hydrophobic amino acids used (e.g., Phe, Leu), all these amino acid-based PEAs reported so far have one common characteristic: they dissolve in organic solvents only and are not water soluble¹⁹.

L-arginine (Arg) is a natural amino acid present in the proteins of all life forms. It carries a positive charge at a physiological pH due to its strong basic guanidine group with an isoelectric point of 10.96 and pKa above 12.5, which is expected to have a strong capability to neutralize negatively charged polymers (proteins/nucleic acids). So L-arginine based poly (ester amide)s (Arg-PEAs) family (Figure 2.2) could achieve two major goals: water solubility and cationic characteristic. The diacid and diol parts of Arg-PEAs can be utilized to not only adjust the physicochemical properties of the Arg-PEA polymers (e.g., hydrophilicity and charge density) but also to convert the resulting Arg-PEA polymers into different physical forms like hydrogels. Some preliminary cell membrane penetrating and DNA transfection tests of 4 types Arg-PEAs have showed that the Arg-PEA/DNA complex could pass through the cell

membrane and transfect SMC A10 cell lines very easily with very low toxicity compared with commercial transfection agents²³, which indicated that the Arg-PEAs could have great potential as gene delivery vector and molecular target agent. However, the details of Arg-PEA monomer and polymer synthesis and physicochemical characterizations have never been reported and discussed, which is very important for developing new types or generations of Arg-PEAs, derivatives and other related polymers. In this paper, we report the details of synthesis of an Arg-PEA family. The synthesis protocols were intensively studied and optimized. The resulting cationic L-Arginine based poly (ester amide)s were characterized by standard physicochemical methods. The cytotoxicity of all the Arg-PEAs was assessed by MTT assay. These data would provide solid physicochemical support for the biological applications of Arg-PEA, such as for small molecules/nucleic acid/protein delivery, molecular targeting agents and tissue engineering scaffolds.

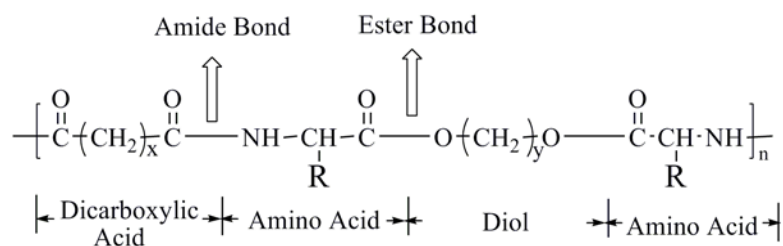


Figure 2.1 Chemical structure of poly (ester amide)

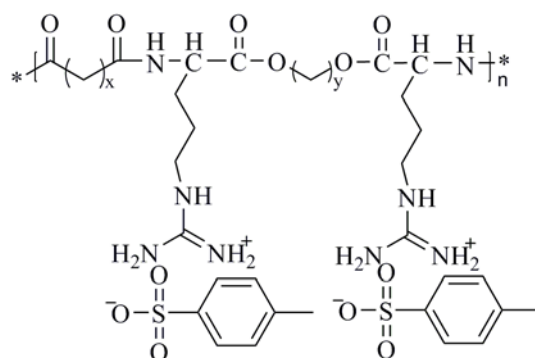


Figure 2.2 Chemical structure of Arg-PEAs: x-Arg-y-S (S stands for toluenesulfonic acid salt), where x is the number of methylene groups between two closest amide groups and y is the number of methylene groups between two closest ester groups.

2.C Experimental

2.C.1 Materials

L-Arginine (L-Arg), p-toluenesulfonic acid monohydrate, fumaryl chloride, succinyl chloride, adipoyl chloride, sebacoyl chloride, ethylene glycol, 1, 4-butanediol, 1,6-hexanediol and p-nitrophenol were all purchased from Alfa Aesar (Ward Hill, MA) and used without further purification. Triethylamine from Fisher Scientific (Fairlawn, NJ) was dried by refluxing with calcium hydride, and then distilled before use. Superfect[®] was purchased from Qiagen. Polyethylenimine (PEI) with a reported weight average molecular weight of 25,000, poly(L-lysine) (PLL)-hydrobromide, ethidium bromide, MTT, Phosphate-buffered saline (PBS, pH 7.4), HEPES were purchased from Sigma (St. Louis, MO). Organic solvents like methanol, toluene, ethyl acetate, acetone, 2-propanol and dimethyl sulfoxide (DMSO) were purchased from VWR Scientific (West Chester, PA) and were purified by standard methods

before use. Other chemicals and reagents if not otherwise specified were purchased from Sigma (St. Louis, MO).

2.C.2 Measurement Methods

The physicochemical properties of the prepared monomer and polymers were characterized by various standard methods. For Fourier transform infrared (FTIR) characterization, the samples were ground into powders and mixed with KBr at a sample/KBr ratio of 1:10 (w/w). FTIR spectra were then obtained with a PerkinElmer (Madison, WI) Nicolet Magana 560 FTIR spectrometer with Omnic software for data acquisition and analysis. ^1H NMR spectra were recorded with a Varian Unity Inova 400-MHz spectrometer (Palo Alto, CA). Deuterated water ($\text{D}_2\text{O}-d_2$; Cambridge Isotope Laboratories, Andover, MA) with tetramethylsilane as an internal standard or deuterated dimethyl sulfoxide ($\text{DMSO}-d_6$; Cambridge Isotope Laboratories) was used as the solvent. MestReNova software was used for the data analysis. Elemental analyses of the synthesized polymers were performed with a PE 2400 CHN elemental analyzer by Atlantic Microlab (Norcross, GA). The thermal properties of the synthesized Arg-PEAs were characterized with a DSC 2920 (TA Instruments, New Castle, DE). The measurements were carried out from -10 to 200 °C at a scanning rate of 10 °C/min and at a nitrogen gas flow rate of 25 mL/min. TA Universal Analysis software was used for thermal data analysis. X-ray diffraction data were obtained from powdered samples with a θ - θ diffractometer (Scintag, Inc., Cupertino, CA) with Cu K α radiation (wavelength 1.5405 Å). The solubility of Arg-PEAs in common organic solvents at room temperature was assessed by using 2.0 mg/mL as a solubility standard to determine whether a Arg-PEA polymer is soluble or not in a solvent. The quantitative solubility of Arg-PEAs in distilled water at room temperature was

measured by adding distilled water step by step until the clear solution was obtained. For the molecular weight measurement, Arg-PEAs were prepared at a concentration of 1 mg/mL in a 0.1% (w/v) LiCl in DMAc solution. The sample molecular weights were determined from a standard curve generated from polystyrene standards with molecular weights ranging from 841.7 kDa to 2.93 kDa that were chromatographed under the same conditions as the samples. The standard curve was generated from a 3rd order polynomial fit of the polystyrene standard molecular weights.

2.C.3 Synthesis of Monomers and Polymers

The general scheme of the synthesis of Arg-PEAs was divided into the following three major steps : the preparation of di-*p*-nitrophenyl ester of dicarboxylic acids (**I**) (Figure 2.3), the preparation of tetra-*p*-toluenesulfonic acid salts of bis (L-arginine), α , ω -alkylene diesters (**II**) (Figure 2.4), and the synthesis of Arg-PEAs (**III**) via solution polycondensation of (**I**) and (**II**) (Figure 2.5). Monomer **I** has been synthesized in our prior studies¹⁹. Monomer **II** is new, and modified procedures were used to accommodate the ionic nature of the L-arginine.

2.C.3.a Synthesis of Di-*p*-nitrophenyl Ester of Dicarboxylic Acids (I)

Di-*p*-nitrophenyl esters of dicarboxylic acids were prepared by reacting dicarboxylic acyl chloride varying in methylene length with *p*-nitrophenol as previously reported^{19,20}. Three saturated and one unsaturated monomers were made; the three saturated diacid monomers were: di-*p*-Nitrophenyl Succate (**NSu**) with $x=2$; di-*p*-Nitrophenyl Adipate (**NA**) with $x=4$; di-*p*- Nitrophenyl Sebacate (**NS**) with $x=8$; and one unsaturated monomer was di-*p*-Nitrophenyl Fumarate (**NF**) with $x=2$. x is the

numbers of methylene group in the diacid. An example of the diacid monomer synthesis is given below. Di-*p*-nitrophenyl succinate (NSu) was prepared in 65 % yield by the reaction of the succinyl chloride (0.15 mol, 16 mL) with *p*-nitrophenol (0.31 mol) in acetone in the presence of triethylamine (0.32 mol). An ice/water mixture bath was used to keep the *p*-nitrophenol and triethylamine mixed acetone solution (400 mL) at 0 °C. Succinyl chloride was diluted in 100 mL of cold acetone before dropped into the above chilled solution with stirring for 2 h at 0 °C and overnight at room temperature. The resulting di-*p*-nitrophenyl ester of succinic acid was precipitated in distilled water, washed completely, and then dried *in vacuo* at room temperature before final recrystallization in ethyl acetate/DMF(4:1, v:v) for three times. The final product is needle-like, colorless crystal.

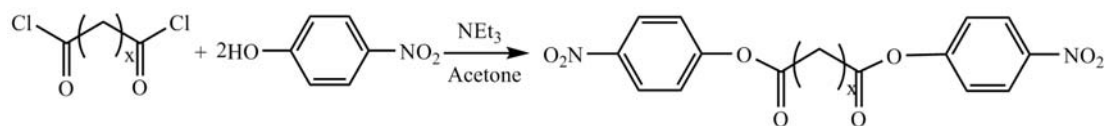


Figure 2.3 Synthesis of monomer I: di-*p*-nitrophenyl ester of dicarboxylic acids

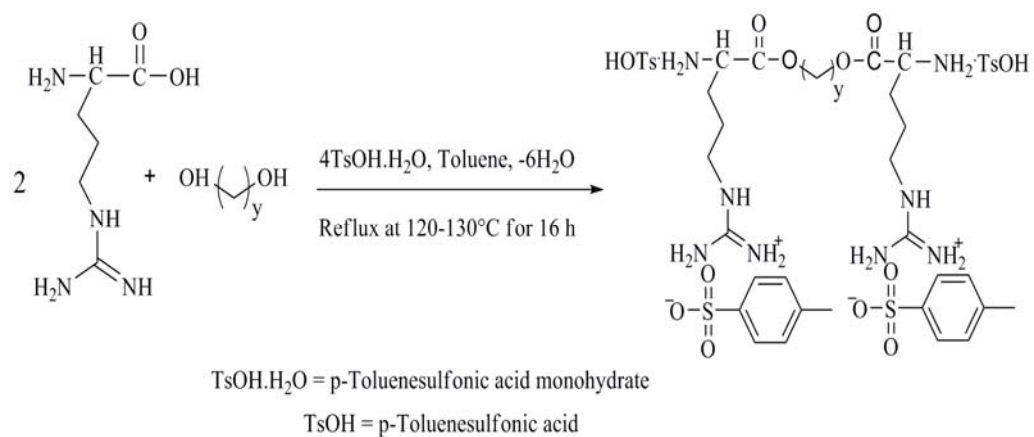


Figure 2.4 Synthesis of monomer II:

Tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) alkylene diesters

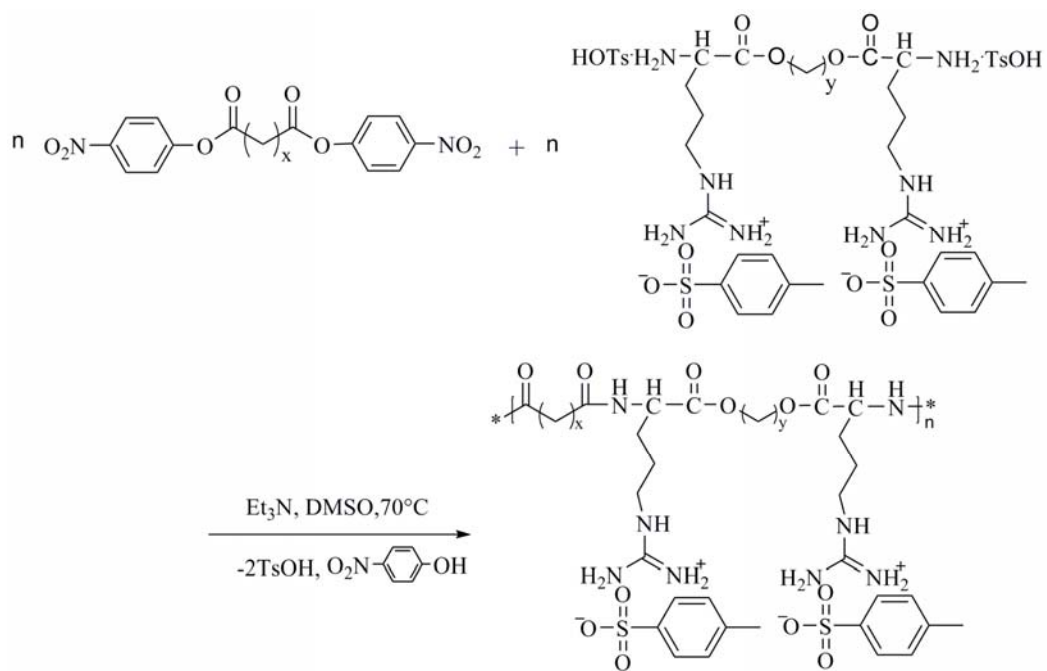


Figure 2.5 Synthesis of Arg-PEAs

2.C.3.b Synthesis of Tetra-*p*-toluenesulfonic Acid Salt of Bis(L-arginine) Alkylene Diesters (II)

Because of the strong positive charge characteristic of L-arginine, the prior PEA non-charged monomer synthesis protocol (e.g., Phe, Leu) was modified. In brief, the amount of *p*-toluenesulfonic acid used for the synthesis of *p*-toluenesulfonic acid salt of L-arginine diester was doubled when compared with the prior synthesis of *p*-toluenesulfonic acid salt of non-ionic hydrophobic amino acids diesters^{19,20}. The need to double the amount of *p*-toluenesulfonic acid in the current case is because of the preferential consumption of the *p*-toluenesulfonic acid by the strong basic guanidine group on L-arginine side chain. For example, L-arginine (0.04 mol) and 1, 4-butanediol (0.02 mol) were directly mixed in a three neck round bottom flask with toluene (400 mL, b.p. 110 °C) with the presence of *p*-toluenesulfonic acid monohydrate (0.082 mol). The solid-liquid reaction mixture was heated to 130 °C and reflux with stirring for 24 hr with 2.16 mL (0.12 mol) of water was generated. The reaction mixture (viscous solid) was then cooled to room temperature. Toluene was decanted. The resulting product was finally purified by dissolving the product in 2-propanol at 75 °C with stirring and then precipitating at 4 °C for three times. The ideal precipitation time is around 12h. 2-propanol was changed every time after precipitating and decanted afterwards, and the white sticky mass was dried in vacuo. The final product was white powder, and obtained in high yields (70~90%). Three types of monomer II were made in this study: tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) ethane diesters, Arg-2-S, $y=2$; tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) butane diesters, Arg-4-S, $y=4$; tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) hexane diesters, Arg-6-S, $y=6$. S indicated that the arg diester monomer was in the *p*-toluenesulfonic acid salt form.

2.C.3.c Synthesis of Arg-PEAs (III) by Solution Polycondensation of I and II Monomers

Arg-PEAs were prepared by solution polycondensation of the monomer **I** (Arg-2-S, Arg-4-S, and Arg-6-S) and monomer **II** (NSu, NA, NS and NF) at a variety of combinations. Such combinations and the resulting Arg-PEAs are summarized in Table 2.1. The saturated Arg-PEAs are labeled as x-Arg-y-S, where x and y are the number of methylene group in diacid and diol, respectively. The unsaturated Arg-PEAs are labeled as x-UArg-y-S, where x and y are the number of CH and CH₂ groups in diacid and diol, respectively. U means the Arg-PEA is unsaturated. An example of the synthesis of 8-Arg-6-S via solution polycondensation is given here. Monomers NS (1.0 mmol) and Arg-6-S (1.0 mmol) in 1.5 mL of dry DMSO were mixed well by vortexing. The mixture solution was heated up to 75°C with stirring to obtain a uniformed mixture. Triethylamine (0.31 mL, 2.2 mmol) was added drop by drop to the mixture at 75°C with vigorous stirring until the complete dissolution of the monomers. The solution color turned into yellow after several minutes. The reaction vial was then kept for 48 hrs at 75°C in a thermostat oven without stirring. The resulting solution was precipitated in cold ethyl acetate, decanted, dried, re-dissolved in methanol and re-precipitate in cold ethyl acetate for further purification. Repeat the purification for 2 times before drying *in vacuo* at room temperature. The final Arg-PEAs are yellow or pale yellow solid powder.

Table 2.1 Arg- PEAs prepared by different combination of monomers

	Arg-2-S	Arg-4-S	Arg-6-S
NSu	2-Arg-2-S	2-Arg-4-S	2-Arg-6-S
NA	4-Arg-2-S	4-Arg-4-S	4-Arg-6-S
NS	8-Arg-2-S	8-Arg-4-S	8-Arg-6-S

2.C.4 Cell Culture and Cytotoxicity

Rat aortic A10 vascular smooth muscle cells (SMC)s obtained from American Tissue Culture Collection (ATCC) were provided by Dr. Bo Liu's lab at Cornell Weill Medical College. The SMCs were grown as recommended at 37°C in 5% CO₂ in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS (Germini, Woodland, CA) and antibiotics.

The cytotoxicity of Arg-PEAs was performed by MTT assay. Cultured SMC were seeded at an appropriate cell density concentration (3,000 cells/well) in 96-well plates and incubated overnight in a 5 % CO₂ incubator at 37 °C. The cells were then treated with various Arg-PEA solutions for 4 h or 48 h. Cells were treated only with normal cell culture media were used as negative control (NC). PEI, PLL-HBr and Superfect[®] treated cells were used as positive control. After 48 h incubation, 15 µL of MTT solution (5 mg/mL) was added to each well, the cell culture plate was incubated for 4 h at 37 °C, 5 % CO₂. After that, the cell culture medium including polymer solution was carefully removed and 150 µL of acidic isopropyl alcohol (with 0.1 M HCl) was added to dissolve the formed formazan crystal. OD was measured at 570 nm (subtract background reading at 690 nm) using a microplate reader. The cell viability (%) was

calculated according to the following equation: Viability (%) = $(OD_{570 \text{ (sample)}} - OD_{690 \text{ (sample)}}) / (OD_{570 \text{ (control)}} - OD_{690 \text{ (control)}}) \times 100\%$, where the $OD_{570 \text{ (control)}}$ represents the measurement from the wells treated with medium only, and the $OD_{570 \text{ (sample)}}$ from the wells treated with various polymers. Triplicates were used in each experiment.

2.C.5 Gel Retardation Assay

The N3014 DNA used here was purchased from New England Lab, and the DNA was visualized by UV illumination (FOTO/UV 300 Transilluminator). The Arg-PEA/DNA complexes were prepared by adding the cationic Arg-PEA polymer aqueous solutions into the solution of the DNA in 20 mM HEPES buffer at pH 7.4. After mixing the two solutions together, it was immediately but slightly vortexed for 2-3 seconds, and then equilibrated at an ambient condition for 30 minutes. The ratio of Arg-PEA to DNA used to prepare the complexes is represented as the weight ratio, and a wide range of weight ratios of Arg-PEA to DNA weight ratio was tested. The Arg-PEA/DNA complexes were analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide (10 ug/mL) in TAE buffer at 100 V for 60 min. N3014 DNA solution without Arg-PEA was used as blank control. The migration of DNA from the Arg-PEA/DNA complex was recorded by a digital camera (Panasonic WV-BP330) installed on the Polaroid MP-4 land camera copy stand.

2.C.6 Fabrication of Arg-PEA/ Polyethylene Glycol Diacrylate (PEGDA) Hybrid Hydrogel

To assess the functionality of the unsaturated Arg-PEAs, hybrid hydrogels from the unsaturated Arg-PEAs were fabricated in an aqueous medium by a photo means.

PEGDA as a co-precursor (molecular weight: 4,000) was synthesized according to a modified procedure of a previously reported method^{24,25}. Several types of Arg-PEA/PEGDA hybrid hydrogel were fabricated. An example for such a fabrication is given here. An unsaturated Arg-PEA (2-UArg-2-S) solution of a concentration of 10.0 % (w/v) was prepared by dissolving 0.5 gram 2-UArg-2-S in 5.0 mL distilled water in a glass bottle. PEGDA ($M_n=4,000$) solution (25 wt %) was then added into the prepared 2-U-Arg-2-S solution at a weight feed ratio of PEGDA to 2-UArg-2-S (i.e., 4.0/1.0). The photoinitiator, 4-(2-hydroxyethoxy) phenyl-(2-hydroxy-2-propyl) ketone (Irgacure 2959) was added to the precursor solution at a concentration of 0.1 % (m/v). The mixed solution was then stirred for 10 minutes at 50°C to ensure a complete dissolution of the photoinitiator. The homogenous, transparent solution was first transferred to a custom-made 20 well Teflon mold (with 500 μ m volume per well) using a micropipette, and then irradiated by a long-wavelength UV lamp (365 nm and 100 W) at room temperature for 10 mins. After photo-gelation, the hydrogel samples were immersed in distilled water at room temperature for 48 h to leach out any unreacted residual chemicals. During this period, distilled water was replaced every 12 hours.

2.D Results and Discussion

As discussed in the introduction part, the main purpose of this paper is to report the synthesis and characterization protocols of Arg-PEAs, so that the new similar systems could be prepared and characterized easily based on the above knowledge. More study about the relationship between polymer structure and biological functions will be reported in the following chapters.

2.D.1 Synthesis of Monomers

Four types of di-*p*-nitrophenyl esters of dicarboxylic acids (**NSu**, **NA**, **NS** and **NF**) were synthesized here as the monomers to react with the tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) alkylene diester monomer (**II**) to provide amide linkage in Arg-PEA backbone. Except the synthesis of the tetra-*p*-toluenesulfonic acid salts of bis(L-arginine) alkylene diesters monomer, the synthesis and characterization of other monomers have been reported previously^{19,20}. The tetra-*p*-toluenesulfonic acid salts of bis (L-arginine) alkylene diesters are newly developed for the first time. The amounts of *p*-toluenesulfonic acid used were the main difference of monomer synthesis between the current Arg-based monomers and other hydrophobic amino acid based monomers. According to our data, the excessive *p*-toluenesulfonic acid was needed because of the strong alkalinity of the guanidine group of Arginine. *p*-toluenesulfonic acid preferred to react with guanidine group first to form a stable salt, then reacted with the amine group of arginine, if there were excessive amounts of *p*-toluenesulfonic acid. Three types of new monomer **II** were prepared: Tetra-*p*-toluenesulfonic acid salt of L-**Arginine** ethane-1,2-diester (**Arg-2-S**), Tetra-*p*-toluenesulfonic acid salt of L-**Arginine** butane-1,4-diester (**Arg-4-S**), and Tetra-*p*-toluenesulfonic acid salt of L-**Arginine** hexane-1,6-diester (**Arg-6-S**). The only difference among these three types of monomer **II** is the methylene chain length (*y*) in the diol part between the two adjacent ester groups: number of CH₂ varies from 2 to 6 from **Arg-2-S** to **Arg-6-S**.

The chemical structures of these 3 types of Arg-based monomer **II** were all confirmed by FTIR and ¹HNMR. As shown in Figure 2.6, the absorption bands of the ester group were observed in the regions ~ 1180 cm⁻¹ (—O—) and ~ 1758 cm⁻¹ (—CO—), and NH vibrations at 3290 cm⁻¹. All the synthesized tetra-*p*-toluenesulfonic

acid salts of bis(L-arginine) alkylene diesters are very moisture sensitive and should be stored under vacuum at room temperature.

The following are some physical and chemical details of the tetra-*p*-toluenesulfonic acid salts of bis(L-arginine) alkylene diesters monomers: **Arg-2-S**: Yield of purified product: 75%. Appearance: amorphous white powder. IR: 1753 cm⁻¹ [-CO-], 1178 cm⁻¹ [-O-]; ¹HNMR (DMSO-d₆, ppm, δ): 1.63[4H, -CH₂-CH₂-CH₂-NH-], 1.82[4H, -OC(O)-CH(NH₃⁺)CH₂-(CH₂)₂-], 2.29[6H, H₃C-Ph-SO₃-], 3.10[4H, -(CH₂)₂-CH₂-NH-], 4.06 [2H, ⁺H₃N-CH(R)-C(O)-O-], 4.39[4H, -(O)C-O-CH₂-], 7.18, 7.53[16H, Ph], 7.69 [10H, -CH₂-NH(NH₂⁺)-NH₂], 8.42[6H, ⁺H₃N-CH(R)-C(O)-O-]; **Arg-4-S**: Yield of purified product: 78%. Appearance: amorphous white powder. IR: 1743 cm⁻¹ [-CO-], 1170 cm⁻¹ [-O-]; ¹HNMR (DMSO-d₆, ppm, δ): 1.52[4H, -OC(O)-CH(NH₃⁺)-CH₂-CH₂-CH₂-NH-], 1.65[4H, -(O)C-O-CH₂-CH₂-], 1.80[4H, -OC(O)-CH(NH₃⁺)CH₂-(CH₂)₂-], 2.29[6H, H₃C-Ph-SO₃-], 3.10[4H, -(CH₂)₂-CH₂-NH-], 4.04 [2H, ⁺H₃N-CH(R)-C(O)-O-], 4.14[4H, -(O)C-O-CH₂-CH₂-], 7.18, 7.53[16H, Ph], 7.71 [10H, -CH₂-NH(NH₂⁺)-NH₂], 8.40[6H, ⁺H₃N-CH(R)-C(O)-O-]; **Arg-6-S**: Yield of purified product: 84%. Appearance: amorphous white powder. IR: 1749 cm⁻¹ [-CO-], 1171 cm⁻¹ [-O-]; ¹HNMR (DMSO-d₆, ppm, δ): 1.34, [4H, -(O)C-O-CH₂-CH₂-CH₂], 1.61, [4H, -(O)C-O-CH₂-CH₂-CH₂], 1.81, [4H, -OC(O)-CH(NH₃⁺)CH₂-(CH₂)₂-], 2.29, [6H, H₃C-Ph-SO₃-], 3.11, [4H, -(CH₂)₂-CH₂-NH-], 4.03, [2H, ⁺H₃N-CH(R)-C(O)-O-], 4.15, [4H, -(O)C-O-CH₂-CH₂-CH₂], 7.15, 7.50[16H, Ph], 7.73[10H, -CH₂-NH(NH₂⁺)-NH₂], 8.43[6H, ⁺H₃N-CH(R)-C(O)-O-]

2.D.2 Synthesis of Arg-PEA Polymers and Their Properties

Arg-PEAs were prepared according to the reaction scheme in Figure 2.5. The yields, glass transition temperature (T_g), molecular weight of repeating unit are given in Table 2.2. The reaction conditions were optimized in terms of reaction temperature and time, catalyst and its concentration, the molar ratio between 2 monomers, monomer concentration. After testing, we found that the optimal polycondensation reaction conditions for the Arg-PEAs are: reaction temperature: 75°C; duration: 48 h, concentration of each monomer: 1.0-1.5 mol/L; the reaction medium: DMSO; catalyst (acid acceptor): NEt₃. The molar ratio of the two monomers (I and II) should be exactly equal to 1.0: 1.0, and the molar ratio between the monomer and acid receptor is suggested to be 1.0: 1.1. The final product yields are high (> 80%) under the optimized reaction conditions.

For the chemical structure identification of all the synthesized Arg-PEAs, their structures were confirmed by both ¹HNMR and FTIR spectra. Figure 2.7 shows the FTIR spectra of 3 Arg-PEAs. The carbonyl bands at 1648–1650 cm⁻¹ (amide I), 1538–1542 cm⁻¹ (amide II), and 1738–1742 cm⁻¹ (ester), and NH vibrations at 3290 cm⁻¹ are typical for all PEAs obtained. Figure 2.8 shows an example of the ¹HNMR spectrum of 2-Arg-2-S.

Table 2.2 Physical and Thermal Characteristics of Arg-PEAs

Polymer	Unit Formula	Unit MW (g/mol)	Charge Density (mol/kg)	T _g (°C)	Polymer Yield
2-Arg-2-S	C ₃₂ H ₄₈ N ₈ O ₁₂ S ₂	800.9	2.497	50±2	80%
2-Arg-4-S	C ₃₄ H ₅₂ N ₈ O ₁₂ S ₂	828.9	2.413	46±2	83%
2-Arg-6-S	C ₃₆ H ₅₆ N ₈ O ₁₂ S ₂	857.0	2.333	39±2	89%
4-Arg-2-S	C ₃₄ H ₅₂ N ₈ O ₁₂ S ₂	828.9	2.413	48±2	90%
4-Arg-4-S	C ₃₆ H ₅₆ N ₈ O ₁₂ S ₂	857.0	2.333	42±2	88%
4-Arg-6-S	C ₃₈ H ₆₀ N ₈ O ₁₂ S ₂	885.0	2.260	38±2	83%
8-Arg-2-S	C ₃₈ H ₆₀ N ₈ O ₁₂ S ₂	885.0	2.260	42±2	91%
8-Arg-4-S	C ₄₀ H ₆₄ N ₈ O ₁₂ S ₂	913.1	2.190	36±2	87%
8-Arg-6-S	C ₄₂ H ₆₈ N ₈ O ₁₂ S ₂	941.1	2.125	34±2	92%

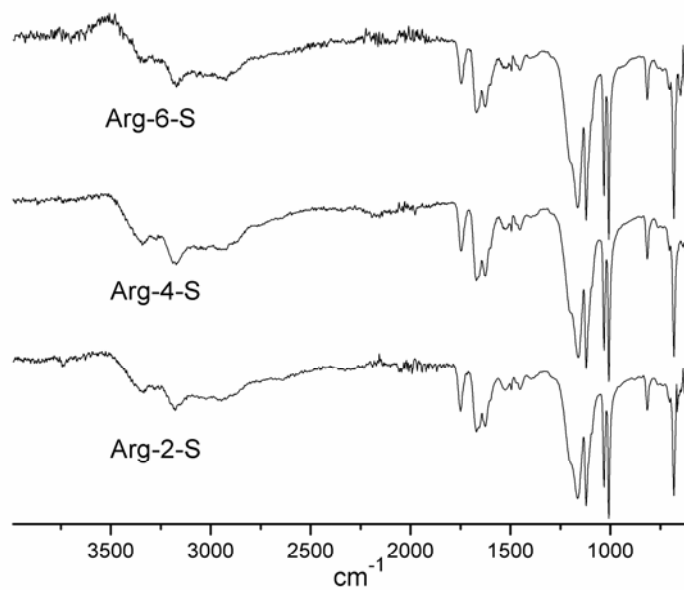


Figure 2.6 FTIR spectra of the tetra-*p*-toluenesulfonic acid salts of bis (L-arginine) diesters

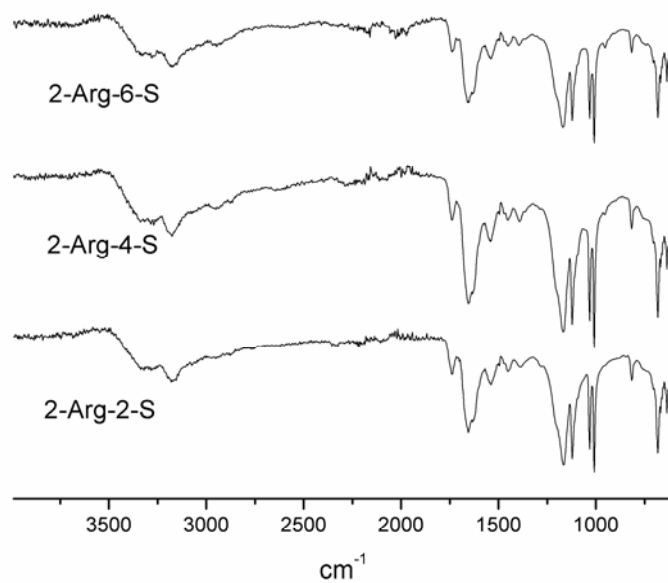


Figure 2.7 FTIR spectra of Arg-PEAs

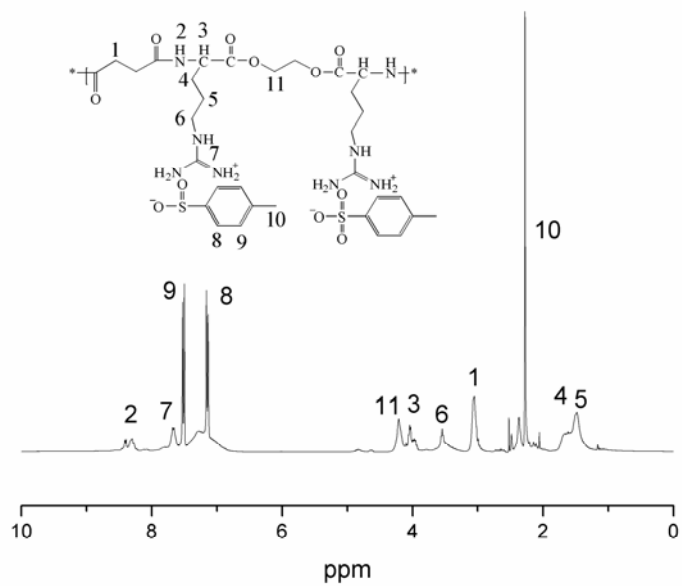


Figure 2.8 HNMR spectra of 2-Arg-2-

For the thermal property of the Arg-PEAs, they do not have melting points (T_m) because all the Arg-PEAs are in the amorphous state which is confirmed by the X-ray diffraction data (see Figure 2.10). The glass transition temperature (T_g) of saturated Arg-PEAs (Table 2.2) ranged between 30-55 °C. An examination for the effect of the number of methylene groups in the diol (y) and diacid (x) parts of the Arg-PEAs revealed that an increase in either x or y led to a lower T_g . For example, if x value was fixed at 2, the T_g decreased from 50 °C to 39 °C when the y value was increased from 2 to 6. The same trend was observed when y value was fixed. This relationship is consistent with non-ionic hydrophobic amino acid-based and many other PEA systems¹⁹, such as the Phe-PEA system, for example, if x value was fixed at 4, the T_g decreased from 59 °C to 49 °C when the y value was increased from 4 to 6. Based on the previous reports, the Phe-based PEAs showed higher T_g than Val or Leu-based PEAs because of the stereo-hindrance effect of the aromatic groups of L-Phe, and it was observed that the T_g value of ionic Arg-PEAs is close to the corresponding non-ionic Phe-PEAs, which could be due to the stereo-hindrance effect of guanidine groups of L-arginine and the cationic property of the guanidine groups. Currently it is not clear which is the main factor affecting the T_g of Arg-PEAs, and further studies would be focused on this area. For the water soluble unsaturated Arg-PEAs, due to the existing of double bonds in the Arg-PEA backbone, much higher T_g values were observed when compared with the saturated Arg-PEAs; this relationship between T_g and instauration in PEA is also consistent with the thermal data of the water insoluble unsaturated Phe-based PEA system²⁰.

The solubility of some Arg-PEAs in water and common organic solvents at room temperature is shown in Table 2.3. Solubility was assessed at 2.0 mg/mL at a room temperature. Due to their strong polar nature, Arg-PEAs tended to dissolve in polar

solvents. All of the Arg-PEAs synthesized were soluble in polar organic solvents like DMSO, methanol or water, but did not dissolve in non-polar or weak polar organic solvents like ethyl acetate or chloroform. And the unsaturated Arg-PEAs showed no solubility difference from the saturated Arg-PEAs. The effect of x and y material parameters on Arg-PEA water solubility (Figure 2.9) revealed that both x and y had a major impact on the water solubility of Arg-PEAs; and an increase in the methylene chain length in either the dicarboxylic acid part (x) or in the diols (y) part reduced the water solubility significantly due to the increasing hydrophobicity. So the water solubility of Arg-PEAs could be used as an index of polymer hydrophilicity/hydrophobicity. By adjusting the x or y, the Arg-PEA polymers' hydrophilicity/hydrophobicity could be fine tuned to meet specific needs.

1.D.3 Molecular Weight and Charge Density of Arg-PEAs

The molecular weight (MW) of 2-Arg-2-S, 4-Arg-2-S and 8-Arg-2-S (Table 2.4) were obtained with the help of MediVas, LLC. The MW data in Table 2.4 indicate that all the three Arg-PEAs had M_n between 12.5 kg/mol and 14.5 kg/mol with narrow polydispersity (PDI) of 1.07 – 1.10. The x or y values did not have any significant impact on MW and PDI of Arg-PEAs. Compared with other amino acid-based PEAs from Phe or Leu, the MW of the Arg-PEA was lower, which may be due to the more complicated and ionic chemical structure of Arg-PEAs.

Table 2.3 Solubility of Arg-PEAs in various solvents^a

	2- Arg- 2-S	2- Arg- 4-S	4- Arg- 6-S	4- Arg- 2-S	4- Arg- 4-S	4- Arg- 6-S	8- Arg- 2-S	8- Arg- 4-S	8- Arg- 6-S
Water	+	+	+	+	+	+	+	+	+
Ethanol	+	+	+	+	+	+	+	+	+
Methanol	+	+	+	+	+	+	+	+	+
1-Propanol	+	+	+	+	+	+	+	+	+
2-Propanol	+	+	+	+	+	+	+	+	+
DMA	+	+	+	+	+	+	+	+	+
DMF	+	+	+	+	+	+	+	+	+
DMSO	+	+	+	+	+	+	+	+	+
THF	-	-	-	-	-	-	-	-	-
Acetone	-	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-	-
Ethyl Acetate	-	-	-	-	-	-	-	-	-
Hexane	-	-	-	-	-	-	-	-	-

^a 2mg/mL was chosen as the solubility standard; + means soluble, - means insoluble

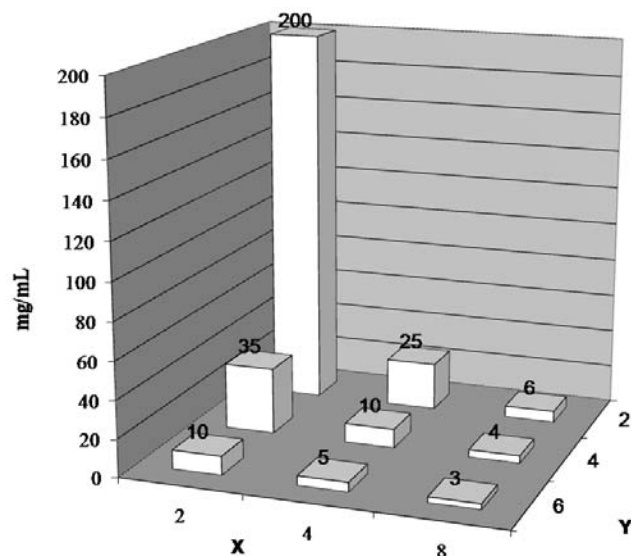


Figure 2.9 Solubility of Arg-PEAs in distilled water

Table 2.4 MW of some Arg-PEAs

Polymer	M _n (kg/mol)	M _w (kg/mol)	PDI
2-Arg-2-S	12.8	13.7	1.07
4-Arg-2-S	14.4	15.9	1.10
8-Arg-2-S	13.2	14.1	1.07

The charge density of Arg-PEAs can be calculated by using guanidine group density (mol/kg) and is shown in Table 2.2. The guanidine group density was found to be controlled by both x and y; and a low x or y led to a higher charge density, and a high x or y resulted in a reduction in charge density. However, the difference in charge density of the current Arg-PEA system was not large; for example, the difference between 2-Arg-2-S (highest charge density) and 8-Arg-6-S (lowest charge density) was around 18%, so we can say all the Arg-PEAs in the present study have a similar cationic strength.

2.D.4 X-Ray Diffraction

Figure 2.10 presents a wide-angle X-ray diffraction diagram of the 3 synthesized Arg-PEAs (2-Arg-2-S, 2-Arg-4-S, and 2-Arg-6-S). Unlike some saturated hydrophobic PEAs, such as the Phe-based PEAs which are semi-crystalline polymers with melting temperatures, all the Arg-PEAs in this study are amorphous, which could be attributed to the salt form in the Arg-PEAs. The large *p*-toluenesulfonic acid counter ion adjacent to the guanidine group could prevent the tight and orderly packing of the Arg-PEA chains required for crystallization.

2.D.5 Cytotoxicity of Arg-PEAs by MTT Assay

Cytotoxicity of Arg-PEAs was evaluated by MTT assay. The MTT system is a simple, accurate, reproducible method of detecting living cells via mitochondrial dehydrogenase activity. An increase in cell number (cell proliferation) results in an increase in the amount of MTT formazan formed and an increase in UV absorbance. Poly (L-lysine) hydrobromide (PLL-HBr), Poly (ethylenimine) (PEI) and Superfect[®] were used as the controls. Figure 2.11 showed an example of the MTT results. The data in Figure 2.11 showed that all the four Arg-PEAs exhibited far better rat A10 SMC cell line viability over a wide concentration range than the three controls, PLL-HBr, PEI and Superfect[®] which showed significant cytotoxicity. Compared to Superfect[®], PLL-HBr and PEI, Arg-PEAs are nontoxic and very safe to the cells even at a high dosage. In addition to the amino acid nature of Arg-PEAs, the relatively lower positive charge density (Table 2.2, guanidine density, ranging from 2.13 to 2.50 mol/kg) of Arg-PEA than the widely studied PEI and PLL-HBr which have a much higher charge density (nitrogen atoms density of 23.3 mol/kg and 4.78mol/kg,

respectively) may be responsible for the much better cell biocompatibility in Arg-PEAs.

2.D.6 Analysis of Polymer/Nucleic Acid Complexes by Gel Retardation Assay

The aim of the gel retardation assay is to preliminarily and visually determine the feasibility of the polyplex formation between cationic Arg-PEAs and anionic nucleic acids; such polyplex formation is the first major step toward the transfection application. The complexes of DNA and cationic Arg-PEAs were prepared at various weight ratios and analyzed by 1% agarose gel electrophoresis. Normally the N/P ratio is used for the characteristic composition of the Polymer/DNA complex under different circumstances. N/P ratio is the ratio of concentrations of total nitrogen atoms (N) of the polycation to the phosphate groups (P) of DNA. In the current study of Arg-PEA system, the only working nitrogen atoms for plasmid DNA are in the guanidine group and three nitrogen atoms only offer one positive charge(due to resonance); therefore, the weight ratio of polymer to DNA was used for examine the effect of Arg-PEAs on their DNA condensation capability. A typical example of an electrophoresis experiments for all Arg-PEA tested is shown in Figure 2.12. In this example, the migration of the DNA fragments in the gel was retarded as the weight ratio of 4-Arg-2-S to DNA increased, demonstrating that the 4-Arg-2-S was able to bind to DNA and completely neutralize its charge when the Arg-PEA to DNA weight ratios were above a critical level. As the ratio of the Arg-PEA to DNA increased further, more gel retardation occurred (Figure 2.12, from lane 5 to lane 2). This method allows an estimation of the appropriate weight ratios of Arg-PEA polymer to DNA required for a complete neutralization of DNA. The detailed effect of the hydrophobic and cationic properties of a variety of Arg-PEAs on their DNA condensing and gene transfection

capability will be presented in a future study. Other polyelectrolyte complex studies in our labs also confirmed that Arg-PEAs could interact with other negatively charged polymers to form stable complex. For example, when Arg-PEAs and hyaluronic acid aqueous solutions were mixed together, they will form stable complex and precipitate from the solutions.

2.D.7 Fabrication of Arg-PEA/PEGDA Hybrid Hydrogel

In order to obtain cationic hydrogels, hybrid hydrogels from both Arg-PEA and PEGDA precursors were fabricated in an aqueous medium by a photo means. Figure 2.13 showed the hydrogel image of 2-UArg-2-S/PEGDA (at 1.0:4.0, w/w). The left image is a completely swollen hydrogel, while the right one is a completely dried hydrogel. The presence and amounts of 2-UArg-2-S in the hybrid hydrogel has been confirmed by elemental analysis. According to the unpublished data, the measured amounts of 2-U-Arg-2-S is very close to the theoretical value, which confirmed that the 2-U-Arg-2-S was almost completely involved the photocrosslinking reaction. This type of cationic Arg-PEA/PEGDA hybrid hydrogel could have many potential applications in the tissue engineering scaffold and drug delivery area.

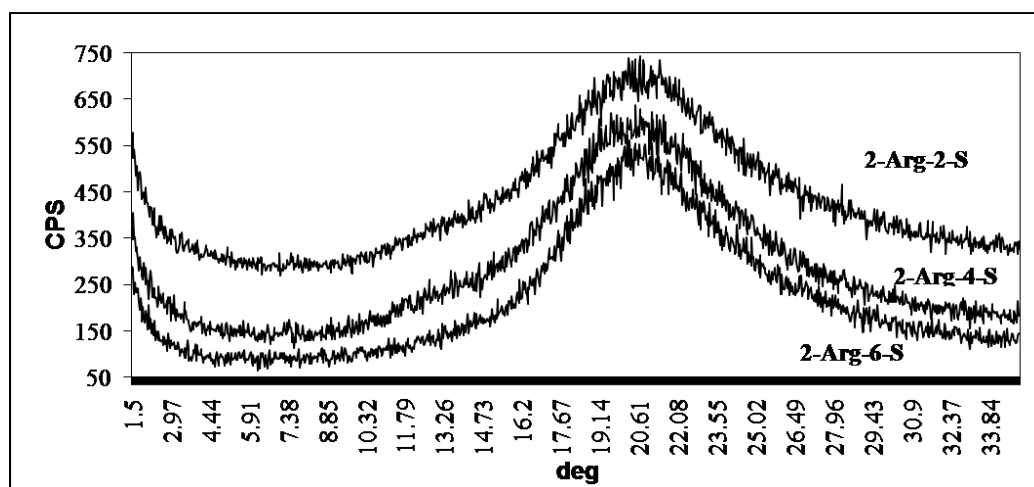


Figure 2.10 X-ray diffraction diagram of Arg-PEAs

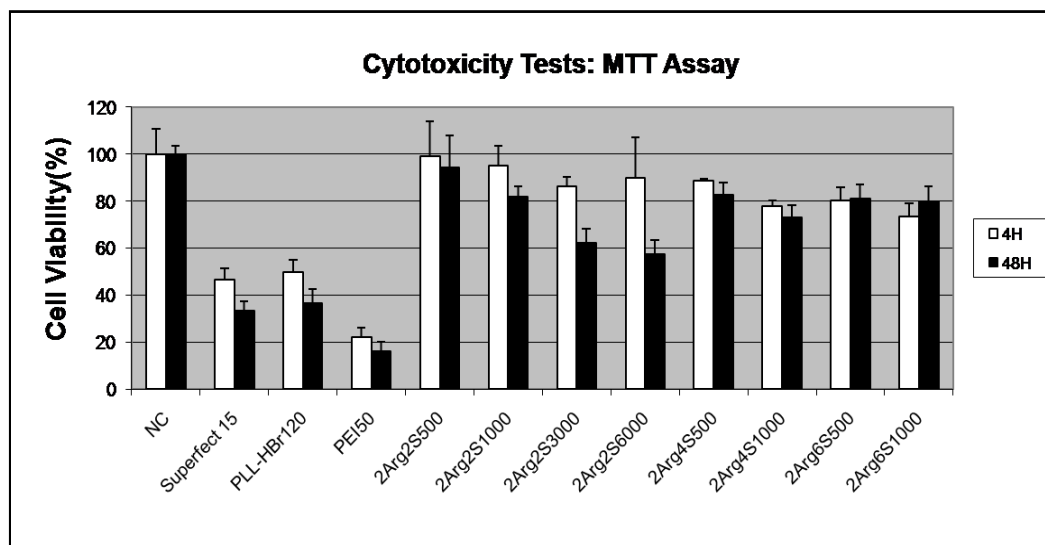


Figure 2.11 Cytotoxicity tests of Arg-PEAs by MTT assay. Negative control (NC) is A10 SMC cell line only without any materials treatment. Various concentrations were tested on Superfect® (SF), PLL-HBr, PEI and Arg-PEA polymers (from 2-Arg-2-S to 2-Arg-6-S). The numbers after the material name indicate the corresponding polymer concentration (ug/mL)

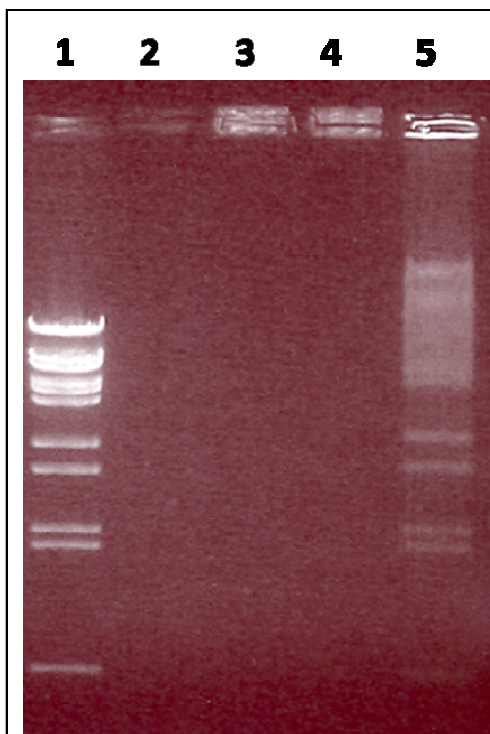


Figure 2.12 Effect of amount of 4-Arg-2-S on the condensation ability of Arg-PEA to DNA by agarose gel electrophoresis: 1.0 μ g N3014S DNA only (lane 1), weight ratio of 4-Arg-2-S: DNA=20, 10, 5, 2 (lane 2, 3, 4, 5, respectively).

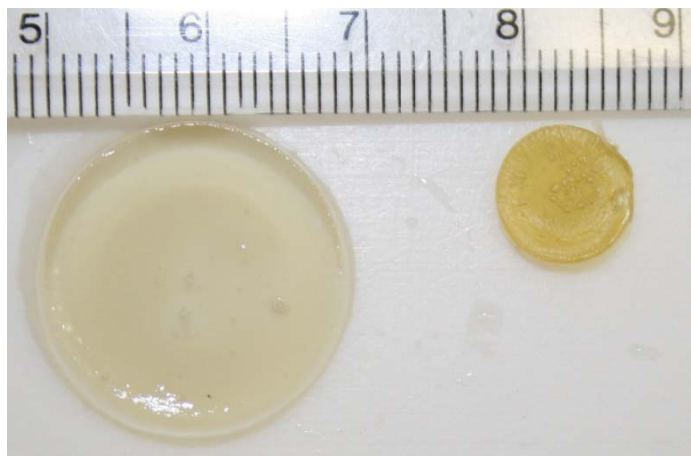


Figure 2.13 Hydrogel image of 2-UArg-2-S/PEG-DA (left: swollen hydrogel; right: dry hydrogel)

2.E Conclusion

A new family of water soluble and cationic L-Arginine-based poly (ester amide)s (Arg-PEAs) have been successfully synthesized and characterized. Various characterization methods have been used to investigate the physicochemical properties of the Arg-PEAs. Arg-PEAs are in the amorphous salt form and proved to be water soluble. The relationship between polymer hydrophilicity and polymer structure was studied, and found that fewer CH₂ units in the Arg-PEA was, the more hydrophilic the Arg-PEA polymer became. The data from the in vitro MTT assay demonstrated that the Arg-PEA polymers are non-toxic to rat A10 SMC cells even at large dosages. The successful hydrogel fabrication from the unsaturated Arg-PEAs in an aqueous medium offered a new type of cationic hydrogel. These Arg-PEAs may have the potential applications for drug delivery and tissue engineering applications.

REFERENCE

1. Langer, R.; Tirrell, D. A., Designing materials for biology and medicine. *Nature (London, United Kingdom)* **2004**, 428, (6982), 487-492.
2. Uhrich, K. E.; Cannizzaro, S. M.; Langer, R. S.; Shakesheff, K. M., Polymeric Systems for Controlled Drug Release. *Chemical Reviews (Washington, D. C.)* **1999**, 99, (11), 3181-3198.
3. Wang, Y.; Ameer, G. A.; Sheppard, B. J.; Langer, R., A tough biodegradable elastomer. *Nature Biotechnology* **2002**, 20, (6), 602-606.
4. West, J. L., Drug delivery: Pulsed polymers. *Nature Materials* **2003**, 2, (11), 709-710.
5. Chu, C.-C., Biodegradable polymeric biomaterials: an updated overview. *Biomaterials* **2007**, 6/1-6/22.
6. Langer, R., Biomaterials in Drug Delivery and Tissue Engineering: One Laboratory's Experience. *Accounts of Chemical Research* **2000**, 33, (2), 94-101.
7. Ma, P. X., Biomimetic materials for tissue engineering. *Advanced Drug Delivery Reviews* **2008**, 60, (2), 184-198.
8. Deng, M.; Wu, J.; Reinhart-King, C. A.; Chu, C.-C., Synthesis and Characterization of Biodegradable Poly (ester amide)s with Pendant Amine Functional Groups and in Vitro Cellular Response. *Biomacromolecules* **2009**, 10, (11), 3037-3047.
9. Zhu, A.; Zhang, M.; Wu, J.; Shen, J., Covalent immobilization of chitosan/heparin complex with a photosensitive hetero-bifunctional crosslinking reagent on PLA surface. *Biomaterials* **2002**, 23, (23), 4657-4665.
10. Huang, N. F.; Patel, S.; Thakar, R. G.; Wu, J.; Hsiao, B. S.; Chu, B.; Lee, R. J.; Li, S., Myotube Assembly on Nanofibrous and Micropatterned Polymers. *Nano Letters* **2006**, 6, (3), 537-542.

11. Kim, K.; Luu, Y. K.; Chang, C.; Fang, D.; Hsiao, B. S.; Chu, B.; Hadjiargyrou, M., Incorporation and controlled release of a hydrophilic antibiotic using poly(lactide-co-glycolide)-based electrospun nanofibrous scaffolds. *Journal of Controlled Release* **2004**, 98, (1), 47-56.
12. Zong, X.; Bien, H.; Chung, C.-Y.; Yin, L.; Fang, D.; Hsiao, B. S.; Chu, B.; Entcheva, E., Electrospun fine-textured scaffolds for heart tissue constructs. *Biomaterials* **2005**, 26, (26), 5330-5338.
13. Zhu, K. J.; Lin, X.; Yang, S., Preparation, characterization, and properties of polylactide (PLA)-poly(ethylene glycol) (PEG) copolymers: a potential drug carrier. *Journal of Applied Polymer Science* **1990**, 39, (1), 1-9.
14. Katayose, S.; Kataoka, K., Water-Soluble Polyion Complex Associates of DNA and Poly (ethylene glycol)-Poly(L-lysine) Block Copolymer. *Bioconjugate Chemistry* **1997**, 8, (5), 702-707.
15. Sawhney, A. S.; Hubbell, J. A., Poly(ethylene oxide)-graft-poly(L-lysine) copolymers to enhance the biocompatibility of poly(L-lysine)-alginate microcapsule membranes. *Biomaterials* **1992**, 13, (12), 863-70.
16. Barrera, D. A.; Zylstra, E.; Lansbury, P. T., Jr.; Langer, R., Synthesis and RGD peptide modification of a new biodegradable copolymer: poly(lactic acid-co-lysine). *Journal of the American Chemical Society* **1993**, 115, (23), 11010-11.
17. Barrera, D. A.; Zylstra, E.; Lansbury, P. T.; Langer, R., Copolymerization and Degradation of Poly(lactic acid-co-lysine). *Macromolecules* **1995**, 28, (2), 425-32.
18. Huang, S. J.; Bansleben, D. A.; Knox, J. R., Biodegradable polymers: Chymotrypsin degradation of a low molecular weight poly(ester-urea) containing phenylalanine. *Journal of Applied Polymer Science* **1979**, 23, (2), 429-37.
19. Katsarava, R.; Beridze, V.; Arabuli, N.; Kharadze, D.; Chu, C. C.; Won, C. Y., Amino acid-based bioanalogous polymers. synthesis, and study of regular poly(ester

amide)s based on bis(alpha -amino acid) alpha ,w-alkylene diesters, and aliphatic dicarboxylic acids. *Journal of Polymer Science, Part A: Polymer Chemistry* **1999**, 37, (4), 391-407.

20. Guo, K.; Chu, C. C.; Chkhaidze, E.; Katsarava, R., Synthesis and characterization of novel biodegradable unsaturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2005**, 43, (7), 1463-1477.

21. Guo, K.; Chu, C. C., Synthesis, characterization, and biodegradation of copolymers of unsaturated and saturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2007**, 45, (9), 1595-1606.

22. Guo, K.; Chu, C. C., Copolymers of unsaturated and saturated poly(ether ester amide)s: synthesis, characterization, and biodegradation. *Journal of Applied Polymer Science* **2008**, 110, (3), 1858-1869.

23. Yamanouchi, D.; Wu, J.; Lazar, A. N.; Craig Kent, K.; Chu, C.-C.; Liu, B., Biodegradable arginine-based poly(ester-amide)s as non-viral gene delivery reagents. *Biomaterials* **2008**, 29, (22), 3269-3277.

CHAPTER 3

WATER SOLUBLE ARGININE POLY (ESTER AMIDE) AS GENE DELIVERY
VECTOR AND INVESTIGATION OF STRUCTURE-FUNCTION
RELATIONSHIP

Jun Wu,¹ Dai Yamanouchi,³ Bo Liu,³ C.C. Chu^{1,2}

¹Department of Biomedical Engineering, Cornell University, Ithaca, NY 14853-4401,
USA

²Department of Fiber Science and Apparel Design, Cornell University, Ithaca, NY
14853-4401, USA

³Department of Surgery, University of Wisconsin, Madison, WI 53705-2275, USA

3.A Abstract

The goals of this study are to evaluate in-depth of a family of water soluble and cationic L-arginine based poly (ester amide)s (Arg-PEAs) as non-viral gene delivery vectors and to thoroughly examine the relationship between Arg-PEAs chemical structure and their gene transfection efficiency and cytotoxicity. The results revealed that changing the number of methylene groups in the diol or diacid unit (x or y) of Arg-PEAs could finely tune the hydrophilic/hydrophobic property of Arg-PEAs, which would greatly affect the polymers' DNA condensing ability and transfection efficiency. The gel retardation assay data show that Arg-PEA's DNA condensing ability increased with increasing either x or y value (i.e., decreasing hydrophilicity). The transfection results obtained from luciferase and GFP assays in rat aortic smooth muscle A10 cell lines (SMC A10) showed that many Arg-PEAs had higher transfection efficiency than the commercial transfection reagent Superfect[®], but at a much lower cytotoxicity. The optimized Arg-PEA/DNA weight ratio (WR) for best transfection performance showed a relationship that the WR decreased with decreasing Arg-PEA hydrophilicity. The zeta potential data of the Arg-PEA/DNA complexes provided a detailed relationship between the DNA condensation and chemical structure of the Arg-PEA, and the data were consistent with the transfection outcomes of Arg-PEA. The *in vitro* MTT assay data indicated that all the Arg-PEA/DNA complexes, over a wide range of dosages, had a minimum cytotoxicity as evident in the cell viability and morphology of SMC A10 and rat smooth muscle primary cells.

3.B Introduction

Gene therapy can be defined as the treatment of human disease by transferring the genetic material into specific cells of patients. During the past several decades, with the fast growing molecular biology techniques, gene therapy technology has been developed rapidly¹⁻⁸. Since the first treatment of patients with gene transfer techniques under the approved FDA protocols in 1990, more than 1000 gene therapy clinical trials have been approved worldwide. However, the successful rate of gene therapy is not very encouraging¹⁻⁷. Based on the reported gene trial results, one of the key limitations is that there have not been safe, efficient and controllable methods for gene delivery⁴⁻¹².

Gene delivery can be mainly accomplished by either virus or non-viral transfer methods. The advantages and disadvantages of viral delivery have been well documented³. Based on the aspect of the clinical safety, the non-viral gene delivery method appears to be the most promising approach. For the reported non-viral gene delivery vectors, most of them can be divided into the following 4 broad categories: water soluble cationic polymers, lipids, dendrimers and nanoparticles. Among them, the water soluble synthetic and natural polycations have attracted the most attentions¹⁰⁻¹⁵. A large number of cationic polymers have been tested for gene delivery. Among them, poly-L-lysine (PLL)¹⁵⁻¹⁷ and polyethylenimine (PEI)^{13, 18-20} have been intensively studied because of their strong interaction with the plasmid DNA, resulting the formation of a compact polymer/DNA complex. Other synthetic and natural polycations developed as non-viral vectors includes polyamidoamine dendrimers^{21, 22} and chitosan²³⁻²⁵, imidazole-containing polymers with proton-sponge effect²⁶⁻²⁸, membrane-disruptive peptides and polymers like polyethylacrylic acid (PEAA)^{29, 30},

poly[alpha-(4-aminobutyl)-L-glycolic acid] (PAGA)³¹, and poly(amino acid) based materials³². However, most of them could not achieve both high transfection efficiency and low toxicity simultaneously.

Recently, many important works were focused on how the polycation's property/structure affect the transfection efficiency or decrease the cytotoxicity of polycations³³⁻⁵³. One well-known example is to introduce the PEG chain to the polycation's backbone or side chain, such as poly (L-lysine)-*b*-PEG and chitosan-*g*-PEG,^{18, 25, 54-58} to form an amphiphilic structure of the polycations. But the results indicated that the introduction of PEG could not help to improve the transfection efficiency in most cases^{18, 25, 54-58}. Putnam et al reported how to improve the transfection efficiency by balancing the side-chain termini of primary amine and imidazole groups¹². Anderson et al prepared around 500 polymers with 70 primary structures to study the structure/properties relationship for gene delivery³⁴. Wong et al did interesting research to explore the relationship between gene delivery efficiency and polymer pendant groups' hydrophilic/hydrophobic properties⁵⁹. Three hydrophobic residues (C4 butyl, C6 hexyl, and C8 octyl) were intensively assessed in terms of their transfection efficiency. Wong et al. found that the introduction of hydrophobic residues would improve the polyplex stability, and hence improved the transfection efficiency. The authors suggested that the improvement of polyplex stability may be related with the conformation rearrangement of polymer during the formation of polyplex, suggested further investigations were needed for the understanding of mechanism of the hydrophobic residues. So more quantities works and precise models/systems are needed for a better understanding of the relationship of polycation structure-function.

Here we designed a precise model, L- arginine based poly (ester amide) (Arg-PEAs, Figure 3.1 and 3.2), to quantitatively study how the hydrophilicity of the polymer backbone could affect their gene delivery; and we proposed the following hypothesis for this study: polycations' hydrophilicity can regulate the interaction between polymer and DNA, and affect the polymer's DNA delivery capability. The amino acid-based poly (ester amide)s have several unique properties for this non-viral gene delivery trial. First, functional poly (ester amide)s (PEAs, Figure 3.2) are newly developed biodegradable and biocompatible polymers having specific functional groups for a variety biomedical applications⁶⁰⁻⁶⁵. The diacid and diol parts of the PEA repeating unit could be precisely controlled and adjusted by selecting different monomers. For example, by changing the number of methylene groups in the diacid or diol segment (x and y), a variety of Arg-PEAs having different hydrophilicity or hydrophobicity could be designed for a systematic and quantitative study of structure – biological function relationship. These Arg-PEAs would have similar other important properties, such as molecular weight and density of functional groups (cationic density). The similarity eliminated many other possible factors that could affect the gene delivery efficiency. Second, L-arginine carries a positive charge at physiological pH due to the guanidino group, a very strong basic group with an isoelectric point of 10.96 and pKa above 12.5, which could have a strong potential to condense negatively charged nuclear acids like plasmid DNA. Thirdly, Arg-based polymers have been shown to enter cells efficiently. Futaki et al. and Mitchell et al have reported in their *in vitro* studies that the poly (L-arginine) can enter the cells more efficiently than other polycationic homopolymers^{66, 67}, however, high molecular weight poly (L-arginine) was found to be very toxic to cells⁶⁶⁻⁶⁹. Recently, preliminary cell membrane penetrating capability and DNA transfection feasibility of a few Arg-PEAs were reported⁸ and showed that the Arg-PEA/DNA complex could pass through

the cell membrane easily and transfect rat aortic SMC A10 cell lines well with very low toxicity when compared with a commercial transfection agent (Superfect®). That preliminary study suggested that the Arg-PEAs could have a great potential as a non-viral gene delivery vector and molecular target agent.

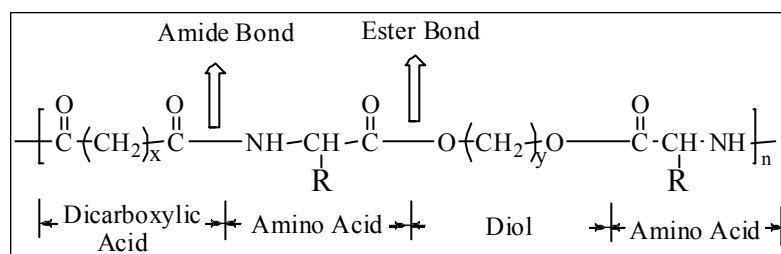


Figure 3.1 General Chemical Structure of an Amino Acid-based Poly (ester amide)

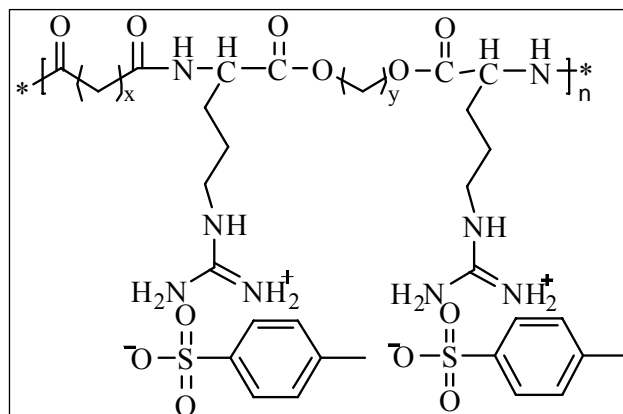


Figure 3.2 Chemical structure of Arg-PEAs: x-Arg-y-S, where x is the number of methylene groups between two adjacent amide linkages and y is the number of methylene groups between two adjacent ester linkages. S stands for toluenesulfonic acid salt.

In this paper, we advanced that preliminary study further by systematically and comprehensively examining the detailed relationship between Arg-PEA polymer chemical structures and their properties and transfection efficiency for the purpose of achieving a better understanding of the relationship between the newly developed water soluble cationic Arg-PEA structure and their biological functions. Vascular smooth muscle cells were chosen to test the transfection efficiency of Arg-PEAs for two reasons. 1) Vascular smooth muscle cells are very important for the formation of vascular lesions, which are major causes of stroke or infarction. 2) Vascular smooth muscle cells (SMC) are very difficult to be transfected with current non-viral gene vectors⁷⁰⁻⁷². Only few papers discussed the gene transfection of SMC via non viral vectors, such as the poly (β -amino ester), PLGA, and cationic phospholipid lipopolyplexes^{73, 74}. Brito et al reported the poly (β -amino ester) and cationic phospholipid lipopolyplexes system for SMC transfection⁷³. They found that the polymers showed low cytotoxicity and better transfection performance than the Lpofectin[®] control. And more new cationic polymers or dendrimers would be needed for SMCs to examine their suitability to achieve high transfection efficiency and low cytotoxicity simultaneously toward SMC.

In this work, a series of Arg-PEAs having different methylene chain length due to different x and y (x=2, 3, 4, 6; y=2, 4, 8) were prepared to test our hypothesis that the hydrophilicity of water soluble polycations like Arg-PEAs can regulate the interaction between polymer and DNA, and subsequently affect the polymer's DNA delivery capability.. These Arg-PEAs would have a wide range of hydrophilicity and charge density for a systematic evaluation of the relationship between chemical structure of the polymers and their properties and transfection efficiency. The findings could help

achieve a better understanding of the structure-function relationship of this new family of water soluble cationic biodegradable Arg-PEAs.

3.C Experimental

3.C.1 Materials

L-Arginine, *p*-toluenesulfonic acid monohydrate, succinyl chloride, adipoyl chloride, sebacoyl chloride, ethylene glycol, 1,3-propanediol, 1, 4-butanediol, 1,6-hexanediol, triethylamine and *p*-nitrophenol were all purchased from Alfa Aesar (Ward Hill, MA) and used without further purification. Polyethylenimine (PEI) with a reported weight average molecular weight (M_w) of 25,000, poly (L-lysine) (PLL)-hydrobromide (M_w 300,000), ethidium bromide, MTT, Dulbecco's phosphate-buffered saline (PBS, pH 7.4), TAE, HEPES and other buffers were purchased from Sigma (St. Louis, MO). Dulbecco's modified eagle medium (DMEM), penicillin–streptomycin (PS, 100 U/mL), trypsin–EDTA (TE, 0.5 % trypsin, 5.3 mM EDTA tetra-sodium), fetal bovine serum (FBS) were obtained from Gibco BRL (Rockville, MD).

Embryonic rat smooth muscle cells A10 and rat aortic smooth muscle primary cells (RSMC primary cell) were obtained from American Type Culture Collection (ATCC, Manassas, VA). DNA size marker N3014 was purchased from New England Lab (Woburn, MA). Qiagen endotoxin-free plasmid Maxi kits and Superfect[®] was purchased from Qiagen (Valencia, CA). Promega Luciferase Assay Kit containing luciferase cell culture lyses reagent and luciferase substrates were obtained from Promega (Madison, WI). Organic solvents like methanol, toluene, ethyl acetate, acetone, 2-propanol and dimethyl sulfoxide (DMSO) were purchased from VWR Scientific (West Chester, PA) and were purified by standard methods before use.

Other chemicals and reagents if not otherwise specified were purchased from Sigma (St. Louis, MO).

3.C.2 Synthesis of Monomers and Polymers

The general scheme of Arg-PEA synthesis was divided into the following three major steps: the preparation of di-*p*-nitrophenyl ester of dicarboxylic acids⁶⁴ (**I**) (Figure 3.3), the preparation of tetra-*p*-toluenesulfonic acid salts of bis (L-arginine), α , ω -alkylene diesters (**II**) (Figure 3.4), and the synthesis of Arg-PEAs (**III**) via the solution polycondensation of monomers (**I**) and (**II**) (Figure 3.5).

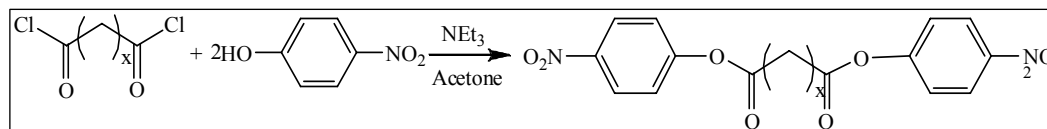


Figure 3.3 Synthesis of Monomer **I**: Di-*p*-nitrophenyl Ester of Dicarboxylic Acid

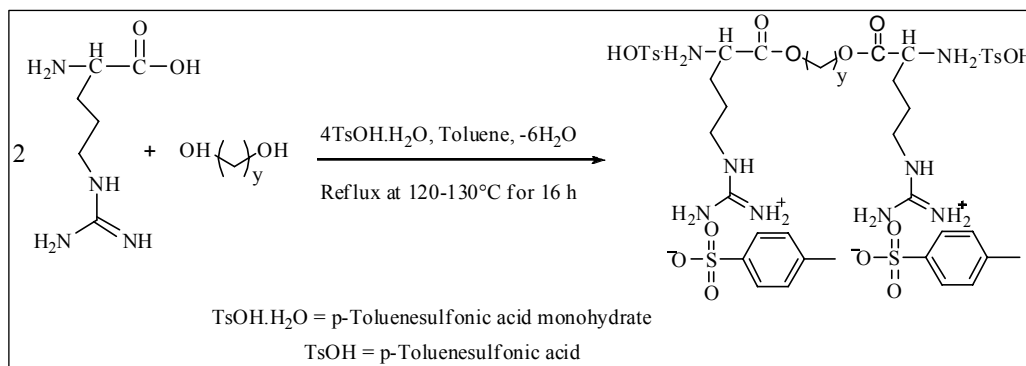


Figure 3.4 Synthesis of Monomer **II**: Tetra-*p*-toluenesulfonic Acid salt of Bis(L-arginine) Alkylene Diesters

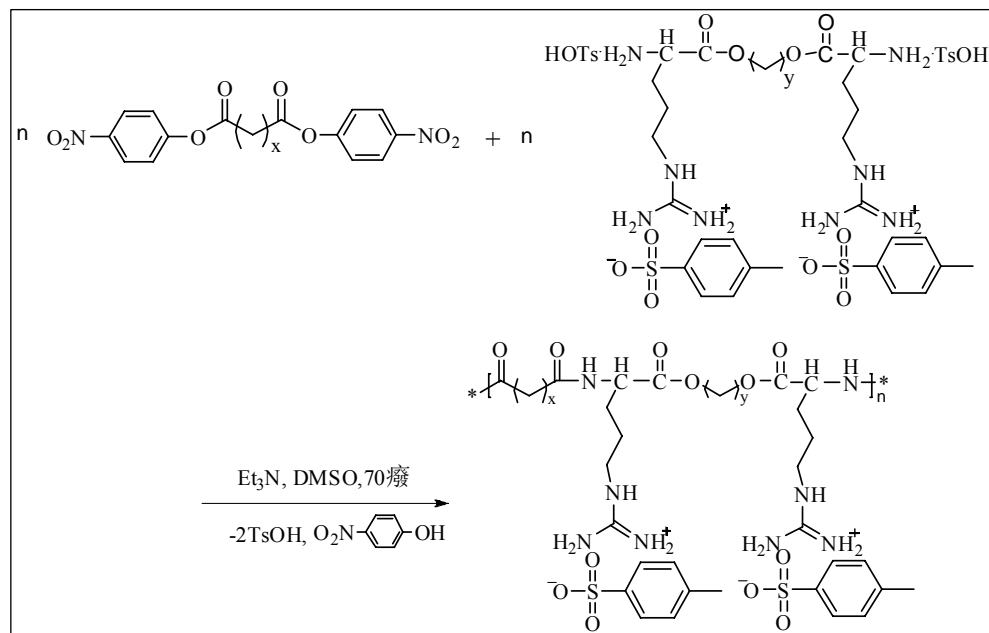


Figure 3.5 Synthesis of Arg-PEAs from monomers **I** and **II**

Di-*p*-nitrophenyl esters of dicarboxylic acids (Monomer **I**) were prepared by reacting dicarboxylic acyl chloride varying in methylene length (*x*) with *p*-nitrophenol described in our previously reported studies⁶⁴⁻⁶⁵. Three monomers were prepared: di-*p*-Nitrophenyl Succinate (**NSu** with *x*=2); di-*p*-Nitrophenyl Adipate (**NA** with *x*=4); di-*p*-Nitrophenyl Sebacate (**NS** with *x*=8). *x* indicates the numbers of methylene group in the diacid. For the synthesis of *p*-toluenesulfonic acid salt of L-arginine diester (**II**), four types of monomer **II**s were prepared in this study: tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) ethane diesters **Arg-2-S** with *y*=2; tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) propane diesters, **Arg-3-S** with *y*=3; tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) butane diesters, **Arg-4-S** with *y*=4; tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) hexane diesters, **Arg-6-S** with *y*=6. Arg-PEAs were prepared by a solution polycondensation of the above (I) and (II) 7 monomers (**NSu**, **NA**, **NS** and **Arg-2-S**, **Arg-3-S**, **Arg-4-S**, **Arg-6-S**) at different combinations in

DMSO solvent and are listed in Table 3.1. The details of Arg-PEA synthesis were given elsewhere. All Arg-PEAs are labeled as x-Arg-y-S, where x and y are the number of methylene groups in diacid and diols, respectively. The molecular weights of some Arg-PEAs (x=2, 4, 6; y=2, 3) were obtained from the help of MediVas, LLC, San Diego, CA.

Table 3.1, Arg-PEAs (x-Arg-y-S) prepared by different combination of diacids and diols building blocks

	Arg-2-S	Arg-3-S	Arg-4-S	Arg-6-S
NSu	2-Arg-2-S	2-Arg-3-S	2-Arg-4-S	2-Arg-6-S
NA	4-Arg-2-S	4-Arg-3-S	4-Arg-4-S	4-Arg-6-S
NS	8-Arg-2-S	8-Arg-3-S	8-Arg-4-S	8-Arg-6-S

3.C.3 Gel Retardation Assay

The Arg-PEA/DNA complexes for a gel retardation assay were prepared by adding the DNA marker (N3014 DNA maker) solution into the Arg-PEA aqueous solutions (in 1X PBS buffer). After mixing the two solutions together, it was immediately vortex for 2-3 seconds, and then equilibrated at an ambient condition for 30 minutes. Arg-PEA/DNA complexes were analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide (10 µg/mL) with TAE buffer at 100 V for 90 min. Total injection volume was 15 µL which consisted of 2 µL blue dye solution, 2 µL DNA marker solution (500 µg/mL), several µL of the Arg-PEA polymer PBS solution and several µL of pure PBS buffer solution. The Arg-PEA solutions must be made freshly or stored at 4 °C before use. The amount of DNA was fixed at 1µg per test.

After mixing all the solutions, the final system was shaken or centrifuged heavily for several seconds. The N3014 DNA marker solution without Arg-PEA was used as a blank control. The N3014 DNA marker was visualized by an UV illumination (FOTO/UV 300 Transilluminator). The migration of DNA from the Arg-PEA/DNA complex was recorded by a digital camera (Panasonic WV-BP330).

3.C.4 Cell Culture

The rat SMC A10 cell lines and rat aortic smooth muscle primary cells (RSMC primary) were grown as recommended at 37 °C in 5% CO₂ in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS and antibiotics. The A10 cell lines were used from passages 6 to 12 and RSMC primary cells were used from passages 3-5. Media was changed every 2 days. Cells were grown to 70% confluence before splitting, harvesting or transfection.

3.C.5 Preparation of Plasmid DNA and Complexes of Arg-PEA/DNA for Zeta Potential Measurement and Transfection Study

The luciferase encoding reporter plasmids COL(-772)/LUC and green fluorescence protein encoding reporter plasmid DNA (GFP) were all provided by Dr. Bo Liu's lab. All plasmids were prepared using Qiagen endotoxin-free plasmid Maxi kits according to the supplier's protocol. The quantity and quality of the purified plasmid DNA was assessed by spectrophotometric analysis at 260 and 280 nm as well as by electrophoresis in 1% agarose gel. Purified plasmid DNA were resuspended in TAE (Tris-acetate-EDTA) buffer and frozen in -20 °C. The DNA solution obtained

had a concentration around 1.5-2.0 mg/mL and was diluted to around 0.5 mg/mL before use.

The Arg-PEA/DNA complexes were prepared by adding the plasmid DNA buffer solution into the freshly prepared Arg-PEA PBS buffer solutions at a room temperature to obtain a desirable Arg-PEA to DNA weight ratio (WR). In this paper, a wide range of WR (from 50 to 6,000) of Arg-PEA to DNA was tested. The mixed solution was immediately and slightly vortex for several seconds and then equilibrated at an ambient condition for 20-30 minutes. All the Arg-PEA solutions and Arg-PEA/DNA complexes were freshly prepared and used within 4 hours.

3.C.6 Zeta Potential Measurements for Arg-PEA/DNA Complexes

The charge property and the relationship of charge-structure of the Arg-PEA/DNA complex were studied by zeta potential measurements. Arg-PEA solutions (2 mg/mL) were prepared by dissolving Arg-PEAs in 1X PBS buffer solution and the solution was filtered (0.45 μm pore size, Whatman[®]) before experiments. The Arg-PEA/DNA complexes were prepared by adding the plasmid DNA buffer solution of pre-determined amounts to the freshly made Arg-PEA PBS buffer solutions (1mL volume total) to obtain desirable Arg-PEA to DNA weight ratio. The mixed solution was immediately and slightly vortex for several seconds, and then equilibrated at an ambient condition for 20 minutes. After that, the zeta potential of the Arg-PEA/DNA complexes was measured at 25 °C by using a Malvern Zetasizer Nano-ZS machine. Zeta potentials were calculated by using the Smoluchowsky model for aqueous suspensions according to manufacturer's protocol. The following Arg-PEAs were selected for this study: 2-Arg-2-S, 8-Arg-2-S and 8-Arg-6-S. Each type of Arg-PEA

was measure with a series of WR of Arg-PEA to DNA. Each sample was measure in triplicate.

3.C.7 Gene Transfection and Luciferase Assay

The complexes formed between plasmid DNA and the Arg-PEAs were assessed for their *in vitro* transfection activity utilizing a transient expression of luciferase reporter in SMC A10 cells. First, the transfection protocol for Arg-PEAs was studied and optimized in terms of cell density, type of buffer for polymer/DNA complex formation, transfection time and media, and temperature. After this optimization, all transfection experiments were carried out according to the optimized protocol.

The details for the optimized transfection protocol for Arg-PEAs are given below. SMC A10 cells were seeded in 0.5 mL complete DMEM (10% FBS, 1% Hepes, 1% penicillin-streptomycin) at 30×10^3 per well in a 24-well plate 24 hours before transfection (70% confluent at transfection). Before transfection, the cell culture media was removed and the cells were washed with PBS buffer twice. Then 1.0 mL warmed serum free DMEM media (without antibiotics) was added into each well. For Superfect[®] and Lipofectamine2000[®], the media was used according to the manufacturer's recommendation. The formulated Arg-PEA/DNA complex solution was then added into each well. The plasmid DNA amount was fixed at 1 μg per well. For the Superfect[®] formulation, 1 μg of plasmid DNA in 60 μL serum-free DMEM were supplemented with 5 μL (3 $\mu\text{g}/\mu\text{L}$) of the Superfect[®] solution in all experiments according to the manufacturer's recommendation. The transfection mixtures were immediately and slightly piped up and down for a few seconds, the cells were transfected for 4 h at 37 °C (5 % CO₂) in an incubator, and then the media solution

was removed. After that, 0.5 mL of complete DMEM (10 % FBS, 1 % Hepes, 1 % penicillin-streptomycin) were added into each well and kept incubated at 37 °C (5 % CO₂) in an incubator. After 48 hours, cells were harvested for luciferase reading.. Triplicate results were obtained in each data point.

Gene expression was then determined by the luciferase activity using a DT 20/20 luminometer (Turner Biosystems, Sunnyvale, CA) with Dual Luciferase Assay System (Promega) according to the manufacturer's instruction. Luciferase assay was performed according to Promega's recommendation. Briefly, cells from each well of a 24-well plate were lysed in 100 µL lysis buffer, transferred to a micro-tube, and then centrifuged at 10,000 g for 2 min. Supernatants were collected and analyzed for luciferase activity. In a typical experiment, 20 µL of supernatant was added to luminometric tubes containing 100 µL of luciferase substrate (Promega). Light emission was measured with a Dual-luciferase detection system for periods of 5 sec, and the relative light units (RLUs) were determined. Triplicate results were used in each experiment. RLUs were normalized to the protein contents of each sample measured by spectrophotometric analysis.

3.C.8 Green Fluorescence Protein (GFP) Assay

To visually confirm the transfection efficiency obtained from the luciferase activity reading, we also transfected the SMC A10 cells with a plasmid DNA encodes for Green Fluorescent Protein (GFP). The transfection protocol was the same as the one used for luciferase assay, except GFP instead of plasmid DNA was used. After 48 h transfection, cells were examined under a fluorescence microscope (Nikon TE2000-U DIC inverted microscope with UV, GFP/FITC and Tx Red filter sets) for any GFP

expression (cells showed green). The cell images were recorded from the random but typical fields of the cell culture wells.

3.C.9 Evaluation of Cytotoxicity of the Arg-PEA/DNA complexes

The evaluation of the cytotoxicity of Arg-PEA/DNA complexes was performed by MTT assay. The cultured SMC A10 cells and RSMC primary cells were seeded at an appropriate cell density concentration (5,000 cells/well) in 96-well plates and incubated overnight in a 5% CO₂ incubator at 37 °C. The cells were, then, treated with various Arg-PEA/DNA complex solutions for 4 h. The media was removed after 4h and complete DMEM was then added. Cells treated only with normal cell culture media were used as the negative control (NC). PEI, PLL-HBr and Superfect® treated cells were used as the positive control. After 48 h incubation at 37 °C and 5% CO₂, 15 µL of MTT solution (5 mg/mL) was added to each well, followed by 4h incubation at 37 °C, 5% CO₂. The cell culture medium including complex solution was carefully removed and 150 µL of acidic isopropyl alcohol (with 0.1 M HCl) was added to dissolve the formed formazan crystal. OD was measured at 570 nm (subtract background reading at 690 nm) using a VersaMax Tunable Microplate reader. The cell viability (%) was calculated according to the following equation: Viability (%) = $(OD_{570(\text{sample})} - OD_{620(\text{sample})}) / (OD_{570(\text{control})} - OD_{620(\text{control})}) \times 100\%$; where the OD_{570(control)} represented the measurement from the wells treated with medium only, and the OD_{570(sample)} from the wells treated with various Arg-PEA/plasmid DNA complexes. Thus, the cell viability was expressed as the percentage of the blank negative control. Triplicates were used in each experiment.

3.C.10 Statistics

Where appropriate, the data are presented as mean \pm standard error of the mean calculated over at least three data points. JMP software (version 8.0, from SAS Company) was used for statistical analysis of data obtained. Significant differences compared to control groups were evaluated by unpaired Student's t-test or Dunnet test at p 0.05, and between more than two groups by Tukey's test with or without one-way ANOVA analysis of variance.

3.D Results and Discussions

3.D.1 Synthesis and Physicochemical Characterization of Arg-PEAs

The details of the synthesis and characterization of all the prepared monomers and polymers of Arg-PEAs have been given elsewhere. In this study, the synthesis protocols of all the Arg-PEAs were optimized. The Arg-PEAs synthesized in this study were in the *p*-toluenesulfonic acid salt form, while all other PEAs from prior reported studies were not in any salt form^{61-64, 75, 76}. This is because of the strong base nature of the guanidine group in L-Arginine. Due to its strong base nature, the *p*-toluenesulfonic acid counter ion, which is normally removed by triethylamine in the last step of polycondensation of amino acid based PEAs from hydrophobic amino acids like phenylalanine and leucine, was tightly bound to the guanidine group. The *p*-toluenesulfonic acid counter ion, however, was found not to adversely affect the DNA binding capability of Arg-PEAs, and all the Arg-PEAs are nontoxic to the cells even at large dosages.

The cationic property (charge density) and some other physicochemical properties of the Arg-PEA polymers synthesized with different x and y are listed in Table 3.2. The data suggested that Arg-PEA has a positive charge density (guanidine groups density was used as an index) ranging from 2.13 to 2.50 mol/kg, while the widely studied PEI and PLL-HBr have a much higher charge density (nitrogen atoms density) of 23.3 mol/kg and 4.78 mol/kg, respectively. However, the guanidine group has a much higher pKa than the amine groups of PEI and PLL-HBr, suggesting a stronger interaction with anionic DNA chain. For the Arg-PEAs, it is rational to assume that the shorter the repeat unit (i.e., smaller x or/and y) was, the higher the charge density would be. The calculated charge density data in Table 3.2, however, show little difference among all the Arg-PEAs studied. For example, the difference between 2-Arg-2-S (highest charge density due to the smallest x and y) and 8-Arg-6-S (lowest charge density) was around 18%; thus, we can state that all the Arg-PEAs synthesized in this study had a similar cationic property and would not be the main factor affecting the observed different gene delivery efficiency of this Arg-PEA system.

Some studies have shown that MW of polycations could affect the gene delivery efficiency^{77, 78}. The data in Table 3.3 show that all the MW of the six Arg-PEAs were within a narrow range (M_n between 12.8 kg/mol and 15.9 kg/mol) and the polydispersity was small (< 1.17 with most < 1.10). Therefore, different x or/and y material parameters did not show an apparent effect on the MW of the Arg-PEAs prepared in this study. Since the MW difference in Arg-PEAs was very small, it was not expected to be the dominant factor affecting the gene delivery data in this study.

In this study, the hydrophilicity of Arg-PEAs was quantitatively evaluated in terms of their solubility in distilled water at room temperature (Figure 3.6); and these

quantitative data were associated with the interaction of Arg-PEAs with DNA and transfection efficiency in the first time. The solubility data in Figure 3.6 indicate that the solubility of Arg-PEAs decreased with an increase in either x or y. For example, the solubility of Arg-PEAs decreased from 200 mg/mL to 10mg/mL as x increased from 2 (2-Arg-2-S) to 8 (8-Arg-2-S) at a constant y=2. A similar solubility – structure relationship was also found with an increase in y at a constant x.

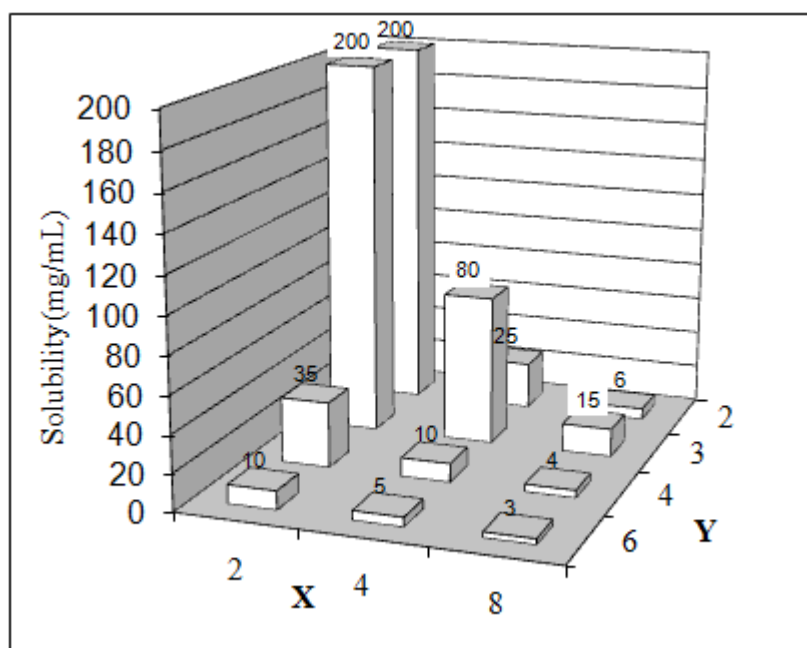


Figure 3.6 Arg-PEA solubility in distilled water

To examine the effect of even vs. odd numbers of methylene chain length, y=3 was chosen for such an illustration. At a constant y=3, the Arg-PEA solubility had the same relationship with an increasing x value, the solubility decreased with an increase in x. However, at a fixed x value, the solubility of Arg-PEA at y=3 was higher than the solubility of Arg-PEAs at y=2 and 4. This *Even–Odd* effect has not been addressed in published gene delivery studies. For the following discussions, we will separate Arg-

PEAs having the odd y value (y=3) from all the rest Arg-PEAs, which have both even x and y values.

The goal of this study was to examine the hypothesis that Arg-PEA chemical structure affects hydrophilicity (water solubility was used as a quantitative index), which, in turn, affects the Arg-PEA/DNA interaction, and affect the transfection efficiency and cytotoxicity. As mentioned in the introduction part, many others' published important studies of gene transfection have focused on the modification of the side chain of existing polymers or making amphiphilic block copolymers to increase the transfection efficiency or cell viability^{12, 34, 54, 59}. Many reports discussed the introduction of PEG chain to the polycation's backbone or side chain to form block copolymer or grafted polymer to change the polycations properties, such as poly (L-lysine) -*b*-PEG and chitosan-*g*-PEG,^{18, 25, 54-58}. Wong et al did intensive study to explore the three types of pendant hydrophobic residues (C4 butyl, C6 hexyl, and C8 octyl)⁵⁹, and more hydrophobic residue would cause less DNA binding stability. However, they did not discuss with details about how the segments in polymer backbone could be adjusted to improve the transfection efficiency and decrease the cytotoxicity. Putnam et al reported the balancing the side-chain termini of primary amine and imidazole groups¹² to regulate the charge properties of the polymers to improve the transfection efficiency and decrease the cytotoxicity. Though the hydrophobic/hydrophilic properties were also modified meanwhile, it was not discussed in details in that report. Anderson et al reported around 500 polymers with 70 primary structures to study the structure/properties relationship for gene delivery³⁴, however, due to the complications and huge amount of the 70 systems, many factors, especially from polymer structure, could not be intensively discussed in details. In this report, we are more interested in how the hydrophobic/hydrophilic properties of

polycations' backbone affect the DNA/polymer interaction and transfection efficiency. So, overall, more quantities works and precise but simple models/systems, especially focusing on the hydrophobic/hydrophilic properties of repeating unit, are needed for a better understanding of the relationship of polycation structure-function.

In this paper, we focused on a few easily defined chemical structure parameters of Arg-PEAs (i.e., x and y) and how the variation in x and y could affect hydrophilicity of Arg-PEAs and their transfection performance and cytotoxicity. The data obtained in this study could advance the understanding of the relationship among chemical structure, hydrophilicity, zeta potential and transfection of the Arg-PEA based biomaterials⁸.

3.D.2 Gel Retardation Assay

Gel Retardation Assay is a widely used method for measuring DNA condensing capability of polymeric transfection candidates. In this study, the main goal is to determine the proper WR of Arg-PEA to DNA required for a completely condensing of DNA during the polyplex formation, the first key step toward non-viral gene transfection. Figure 3.7 exhibits all the electrophoresis data for the Arg-PEA/DNA complexes. These results demonstrated the DNA condensation capability of Arg-PEAs, and provided the basic formulation information for subsequent transfection experiments. Most important of all, the electrophoresis data showed that different types of Arg-PEAs (in terms of x and y parameters) required different amounts of Arg-PEAs for a complete DNA condensation as indicated by the different WR.

Table 3.2 Arg-PEA Properties

Polymer	Unit Formula	Unit MW (g/mol)	Charge Density (mol/kg)	Polymer Yield
2-Arg-2-S	C ₃₂ H ₄₈ N ₈ O ₁₂ S ₂	800.9	2.497	80%
2-Arg-3-S	C ₃₃ H ₅₀ N ₈ O ₁₂ S ₂	814.9	2.454	83%
2-Arg-4-S	C ₃₄ H ₅₂ N ₈ O ₁₂ S ₂	828.9	2.413	83%
2-Arg-6-S	C ₃₆ H ₅₆ N ₈ O ₁₂ S ₂	857.0	2.333	89%
4-Arg-2-S	C ₃₄ H ₅₂ N ₈ O ₁₂ S ₂	828.9	2.413	90%
4-Arg-3-S	C ₃₅ H ₅₄ N ₈ O ₁₂ S ₂	842.9	2.372	87%
4-Arg-4-S	C ₃₆ H ₅₆ N ₈ O ₁₂ S ₂	857.0	2.333	88%
4-Arg-6-S	C ₃₈ H ₆₀ N ₈ O ₁₂ S ₂	885.0	2.260	83%
8-Arg-2-S	C ₃₈ H ₆₀ N ₈ O ₁₂ S ₂	885.0	2.260	91%
8-Arg-3-S	C ₃₉ H ₆₂ N ₈ O ₁₂ S ₂	899.0	2.225	83%
8-Arg-4-S	C ₄₀ H ₆₄ N ₈ O ₁₂ S ₂	913.1	2.190	87%
8-Arg-6-S	C ₄₂ H ₆₈ N ₈ O ₁₂ S ₂	941.1	2.125	92%

Table 3.3 Molecular Weight of Arg-PEAs

Polymer	M _n (kg/mol)	M _w (kg/mol)	PDI
2-Arg-2-S	12.8	13.7	1.07
2-Arg-3-S	13.5	14.5	1.08
4-Arg-2-S	14.4	15.9	1.10
4-Arg-3-S	14.5	16.0	1.11
8-Arg-2-S	13.2	14.1	1.07
8-Arg-3-S	15.9	18.5	1.17

In order to have a better quantitative comparison of the DNA condensation capability of Arg-PEAs, the minimum WR of Arg-PEA to DNA that could completely condense DNA was selected and compared (Figure 3.8). For examples, 2-Arg-2-S needed a minimum WR of 50 to completely condense the DNA marker; while 8-Arg-2-S needed a minimum WR of 5 for a complete condensation. This difference in minimally required WR is believed to be mainly attributed to different levels of hydrophilicity/hydrophobicity of Arg-PEAs since the calculated charge density difference between these 2 Arg-PEAs was relatively small, e.g., the largest difference in charge density was 18% between 2-Arg-2-S and 8-Arg-6-S. The data in Figure 3.8 show that if x or y was fixed at 2, the minimally required WR was reduced with an increase in y or x, respectively. This relationship, however, did not hold at a larger fixed x or y value (such as x=8 and y=6). For example, at a fixed x=8, an increase in y value from 2 to 6 led to an increase in the minimally required WR. It is not clear what the reason behind this observation is. It may be attributed to the shift of the balance of hydrophilicity and hydrophobicity or polymer conformation at this higher x and y

values. Our trend at low fixed x or y is different from the trend reported by Wong et al, who explored three types of pendant hydrophobic residues (C4 butyl, C6 hexyl, and C8 octyl)⁵⁹ and found more hydrophobic residue would cause less DNA binding stability. This trend difference could be caused from many aspects: 1, our system is changing the length of CH_2 units in the backbone unit, while Wong et al's system is for side chain; 2, two polycation systems are significantly different in the aspects of polymer structure and functional groups and Wong et al used poly(methacryloxysuccinimide) as backbone, which was not biodegradable.. However, we estimate that for Arg-PEA system with very large x and y (larger or equal to 8), the trend would be same as trend of Wong's system.

A comparison of the minimally required WR of Arg-PEA to DNA for a complete condensation (Figures 3.7 & 3.8) with the solubility data of Arg-PEAs (Figure 3.6), we can conclude that the Arg-PEAs' hydrophilicity (in terms of solubility) can greatly affect the DNA condensing capability.

We also found that the Arg-PEA buffer solutions, if stored at 4 °C, could retain their DNA condensing capability for around 2 months, suggesting there was no obvious structure change or degradation of Arg-PEAs in the buffer solution at 4 °C. And it is very important to recognize that a complete Arg-PEAs dissolution, precise polymer concentration and volume are critical for reproducible data. In this study, the Arg-PEA polymers must be dissolved completely and the volume should be in the range of 2-5 μL to avoid any possible experimental errors. Some Arg-PEAs have a very low water solubility and would take a very long time for a complete dissolution. If the polymers solutions were not properly prepared, the results would be different.

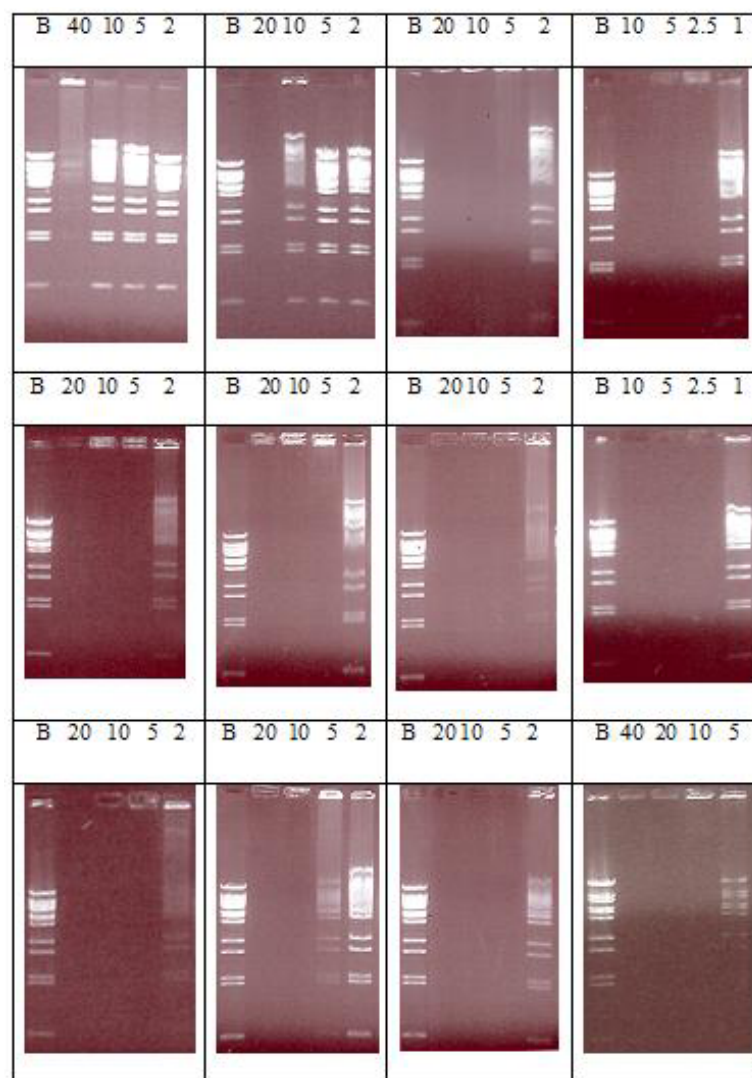


Figure 3.7 Gel retardation assay to show the effect of hydrophobic block length (x or y) of Arg-PEAs on the condensation ability to DNA: B means blank, (only 1 μ g N3014S DNA, no Arg-PEA); the other column numbers are the WR of Arg-PEA to DNA. The Arg-PEAs are: 2-Arg-2-S, 2-Arg-3-S, 2-Arg-4-S, 2-Arg-6-S (first row, from left to right); 4-Arg-2-S, 4-Arg-3-S, 4-Arg-4-S, 4-Arg-6-S (second row, from left to right); 8-Arg-2-S, 8-Arg-3-S, 8-Arg-4-S, 8-Arg-6-S (third row line, from left to right)

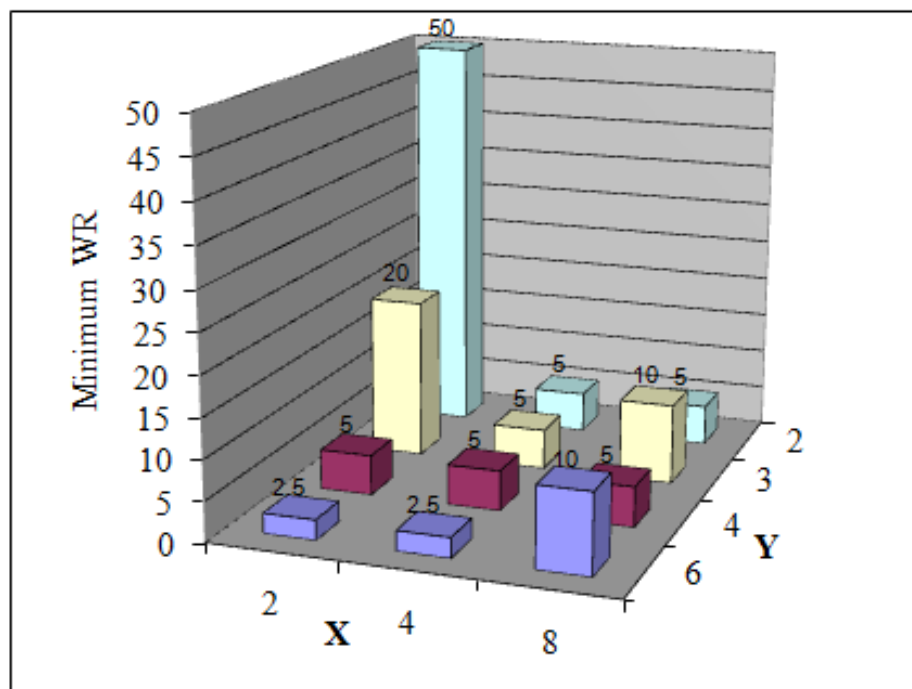


Figure 3.8 The minimum weight ratio (WR) of Arg-PEA to DNA required to completely condensing DNA as a function of methylene chain length in diacid (x) and diol segments (y) of Arg-PEA polymers

3.D.3 Transfection Efficiency

In this paper, the plasmid DNA that encodes for a firefly luciferase driven by a collagen promoter was used. By measuring luciferase activities in cell lysates, which in this case is mainly determined by the amount of DNA transferred into the cells, we compared the transfection efficiency of Arg-PEAs with a commercial transfection agent, Superfect[®] for examining the transfection feasibility of Arg-PEAs.

In any transfection protocol development, cell density, transfection time, transfection temperature, transfection media and buffer types are important parameters for optimization to achieve the best transfection data. In the Arg-PEA/DNA system, the optimized transfection protocol of a particular 2-Arg-3-S/DNA system was: transfection time: 3-4 h; transfection temperature: 37 °C; transfection media: serum free DMEM media without antibiotics; buffer for Arg-PEA/DNA: HEPES (20 mM) or PBS buffer (1X); cell density: 20,000-30,000 per well for 24-well cell culture plate. At this optimized condition, it was observed that the luciferase activity could reach the peak value over a range of WR of Arg-PEA to DNA.

Figure 3.9 showed an example of the transfection results from three types of Arg-PEA/DNA at various WR: 2-Arg-3-S, 4-Arg-3-S and 8-Arg-3-S. PEI was used here as the polymer control and the transfection efficiency was very poor compared with Lipofectamine2000 and Arg-PEAs. The data show that all the Arg-PEA/DNA could show some transfection capability over a very broad WR range, and each type of Arg-PEA/DNA showed a peak transfection at a specific WR. For example, the 8-Arg-3-S/DNA showed transfection capability over WR from 100 to 1000, but its highest

transfection capability was around WR of 200. For the 2-Arg-3-S/DNA system, the peak transfection, however, occurred at WR 2,000.

These transfection results also showed that the WR of Arg-PEAs/DNA to reach the optimum transfection efficiency (Figure 3.9) was much higher than the minimal WR required to completely condense DNA in the electrophoresis data (Figure 3.7). The transfection agent's DNA condensation capability is known to have a profound effect on the subsequent gene delivery efficiency, but is not the only factor that is responsible for the outcome of gene delivery efficiency. This may be attributed to the need of excess amounts of Arg-PEAs required to achieve not only a stable Arg-PEA/DNA complex system in the transfection media but also provided additional cationic charge to the Arg-PEA/DNA complex for its proper penetration into the cells membranes. The larger dosage of Arg-PEA required for the optimized transfection, however, didn't impose any adverse cytotoxicity as described later.

To compare the transfection efficiency of all the Arg-PEAs, the highest or peak RLU/mg (relative light unit/mg) of each polymer was selected and normalized against the RLU/mg value of the commercial control (Superfect[®]), i.e., setting the RLU/mg value of the control at 100 (Figure 3.10). This normalization process removed the batch to batch variation. The normalized transfection data in Figure 3.10 showed that most of these Arg-PEAs had comparable or better transfection efficiency (i.e., those Arg-PEAs having 100 or greater normalized values) than the commercial transfection reagent Superfect[®]. Those Arg-PEAs having lower x and y values (2 and 3) were the most favorable for higher transfection efficiency, while high y value, especially y=6, is not favorable for high transfection. Arg-PEAs having both high x and y (e.g., x=8, y=6) exhibited the lowest transfection.

In this Arg-PEA system, the polymer DNA condensing capability was evaluated by the minimum WR required for a completing DNA condensation, i.e., a smaller minimum WR suggested a higher condensing capability. However, the Arg-PEA polymer with the highest DNA condensing capability (expressed in terms of the smallest minimal WR required) did not show the best transfection. For example, the 8-Arg-6-S showed a much lower transfection capability than 8-Arg-3-S, even though 8-Arg-6-S had a better DNA condensing capability. This may be attributed to the fact that the Arg-PEA that had the strongest DNA binding capability may not easily release DNA after entering cells.

Figure 3.11 showed the WR of all the Arg-PEAs that achieved the highest transfection efficiency. The data in Figure 3.11 show that the WR required for achieving the highest transfection was reduced with an increase in x or y. This relationship of WR for optimal transfection vs. x and y material parameters of Arg-PEAs (Figure 3.11) was very consistent with the relationship between Arg-PEA water solubility (hydrophilicity) vs. x and y material parameters of Arg-PEA (Figure 3.6). For example, from 2-Arg-2-S to 8-Arg-2-S, the WR required for the highest transfection was reduced from 2,000 to 200, while the solubility of these two Arg-PEAs was also decreased from 200 mg/mL to 10 mg/mL. These data suggested that less hydrophilic of the Arg-PEA was, the less Arg-PEA macromolecules were needed to form a stable and efficient complex with DNA for a better transfection.

3.D.4 GFP Expression

To visually confirm the transfection efficiency obtained from the luciferase activity data, SMC A10 cells were transfected by plasmid DNAs encoding for green fluorescent protein (GFP). Two days following such a transfection, the SMC were examined under a fluorescence microscope for their GFP expression (SMC show green). Figure 3.12 shows GFP plasmid DNAs were successfully expressed inside SMCs as commercial transfection agent Superfect[®] did.

3.D.5 Zeta Potential Measurements for DNA/Arg-PEA Complex

The Zeta potential measurement was used to study the charge property and the charge-structure relationship of the Arg-PEA/DNA complex. Figure 3.13 showed the zeta potentials of 3 types of Arg-PEA/DNA complexes as a function of the ratio of Arg-PEA to DNA as well as the type of Arg-PEAs. For example, the data in Figure 3.13(b) (8-Arg-2-S) could be divided into 3 regions, depending on the ratio of Arg-PEA to DNA. As the weight ratio of Arg-PEA to DNA increased, the zeta potential of the complex increased (from negative to positive), suggesting that as more Arg-PEAs added into the DNA, the charge property of the complex changed from negative to positive. A further increase in the weight ratio of Arg-PEA to DNA, the zeta potential of the complex reached a peak, (WR is around 200), and a further increase in the WR resulted in a reduction in zeta potential of the complex. The WR with a peak zeta potential suggests that the Arg-PEA/DNA complex must be in the most stable state, and should be the optimal condition for gene transfection.

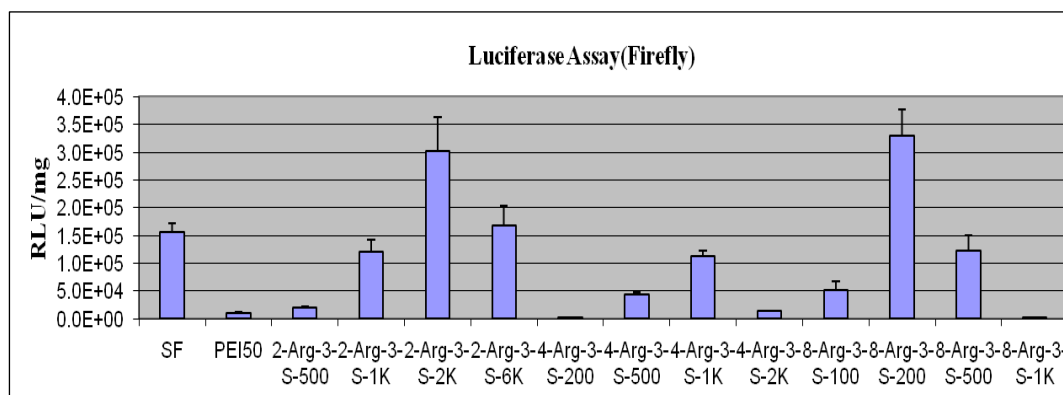


Figure 3.9 Transfection efficiency of Arg-PEA/DNA complexes expressed by firefly luciferase activity. Plasmid DNA used were COL (-772)/Luc. Superfect® (SF) was tested with the optimum weight ratio of SF to DNA. Various WR of Arg-PEA to DNA (indicating by the numeric # after sample labels) were tested.

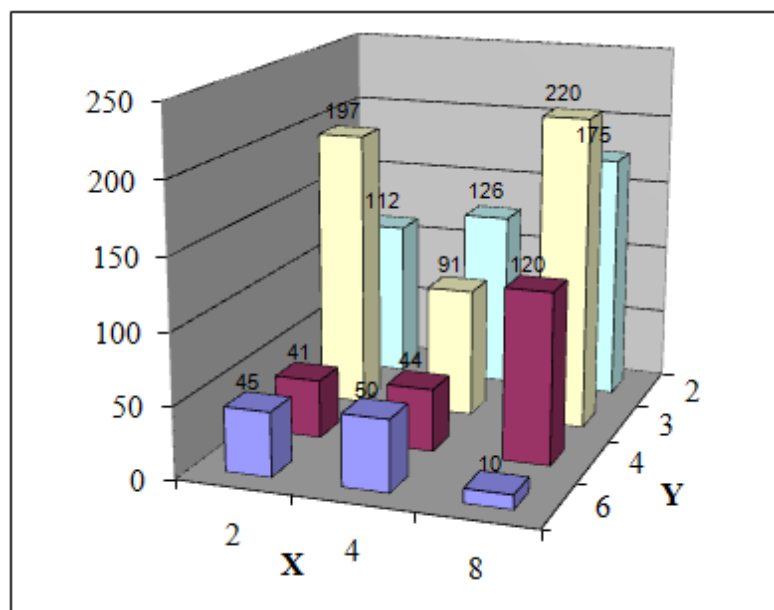


Figure 3.10 the effect of x and y material parameters of Arg-PEAs on the normalized transfection efficiency against a commercial transfection agent, Superfect[®] by setting the RLU of Superfect as 100. Those Arg-PEAs having greater than 100 have better transfection efficiency than the commercial Superfect agent.

A comparison of these WR vs. zeta potential data with the effect of WR on transfection data described previously (e.g., Figure 3.9), the WR at the peak zeta potential coincided with the WR that showed the best transfection efficiency. Therefore, this relationship between the zeta potential of the Arg-PEA/DNA complex with the weight ratio of Arg-PEA to DNA provides a very solid illustration of the charge property and structure change of the Arg-PEA/DNA complex, and the data trend was very consistent with transfection (Figures 3.9 and 3.11) and electrophoresis data (Figures 3.7 and 3.8).

The zeta potential data of these 3 types of Arg-PEAs also illustrate the effect of x and y in Arg-PEAs on their condensation capability with DNA. For example, 2-Arg-2-S (Figure 3.13 (a)) required much higher amounts (WR 2,000) of Arg-PEA than 8-Arg-2-S (WR 200) did (Figure 3.13 (b)) to reach the peak zeta potential. The magnitude of the zeta potential peak also depended on x and y of the Arg-PEAs. For example, 8-Arg-2-S/DNA complex had the largest peak zeta potential (Figure 3.13 (b)), while the 8-Arg-6-S/DNA had the smallest peak zeta potential (Figure 3.13 (c)). This magnitude of peak zeta potential was found to be related to the transfection efficiency. For example, among the 3 Arg-PEA polymers tested (2-Arg-2-S, 8-Arg-2-S, 8-Arg-6-S), the largest peak zeta potential found in 8-Arg-2-S/DNA at WR 200 (Figure 3.13 (b)) coincided with its highest transfection efficiency among the 3 polymers, while the smallest peak zeta potential found in 8-Arg-6-S/DNA complex at WR 100 showed the lowest efficiency (Figure 3.10).

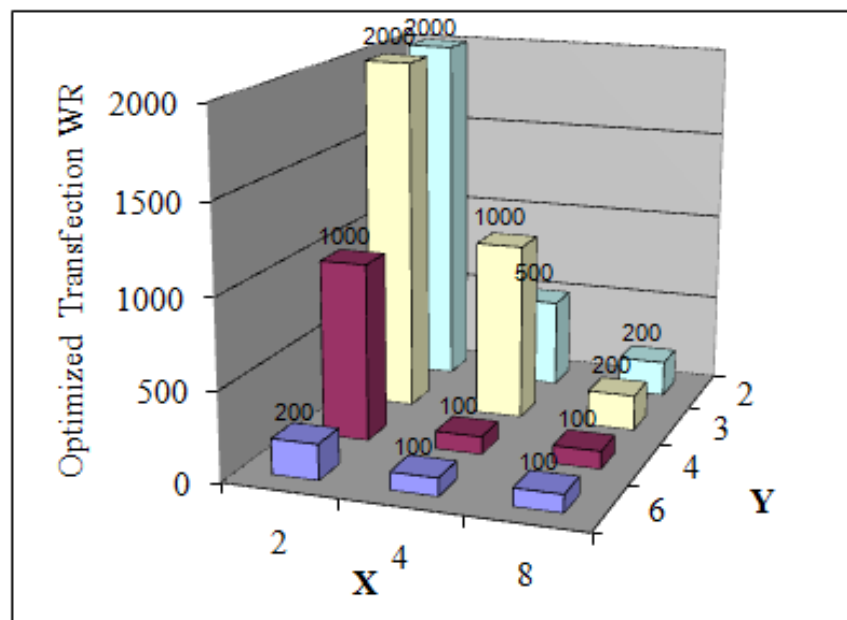


Figure 3.11 The effect of x and y material parameters of Arg-PEAs on their weight ratios of Arg-PEA to DNA (WR) that achieved the highest transfection efficiency.

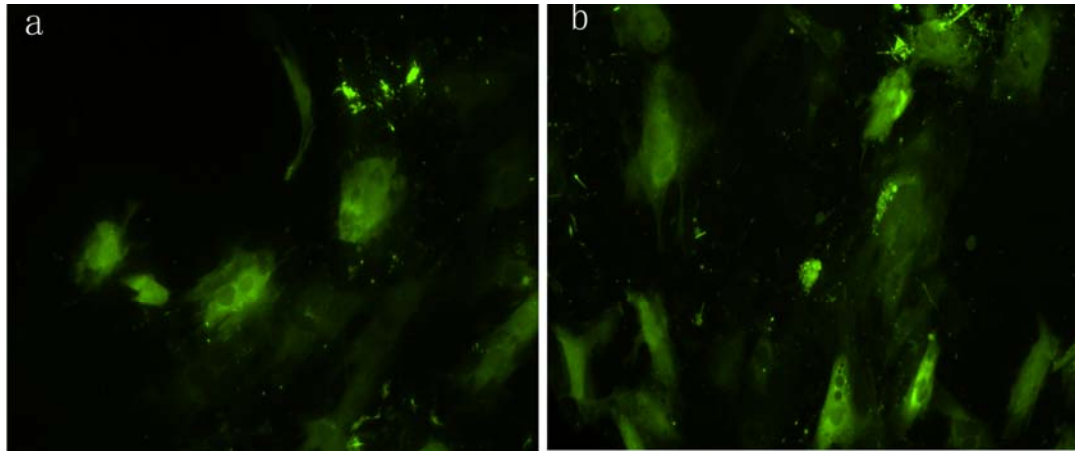


Figure 3.12 Fluorescence microscopic view (10X) of the GFP expression from transfected SMC A10 cells under. Green cells are cells successfully transfected with GFP DNA. (a) SMC A10 cells were transfected by Superfect®. (b) SMC A10 cells were transfected by Arg-PEA (2-Arg-3-S, WR=2,000). 4hr transfection and the images were taken after 48h

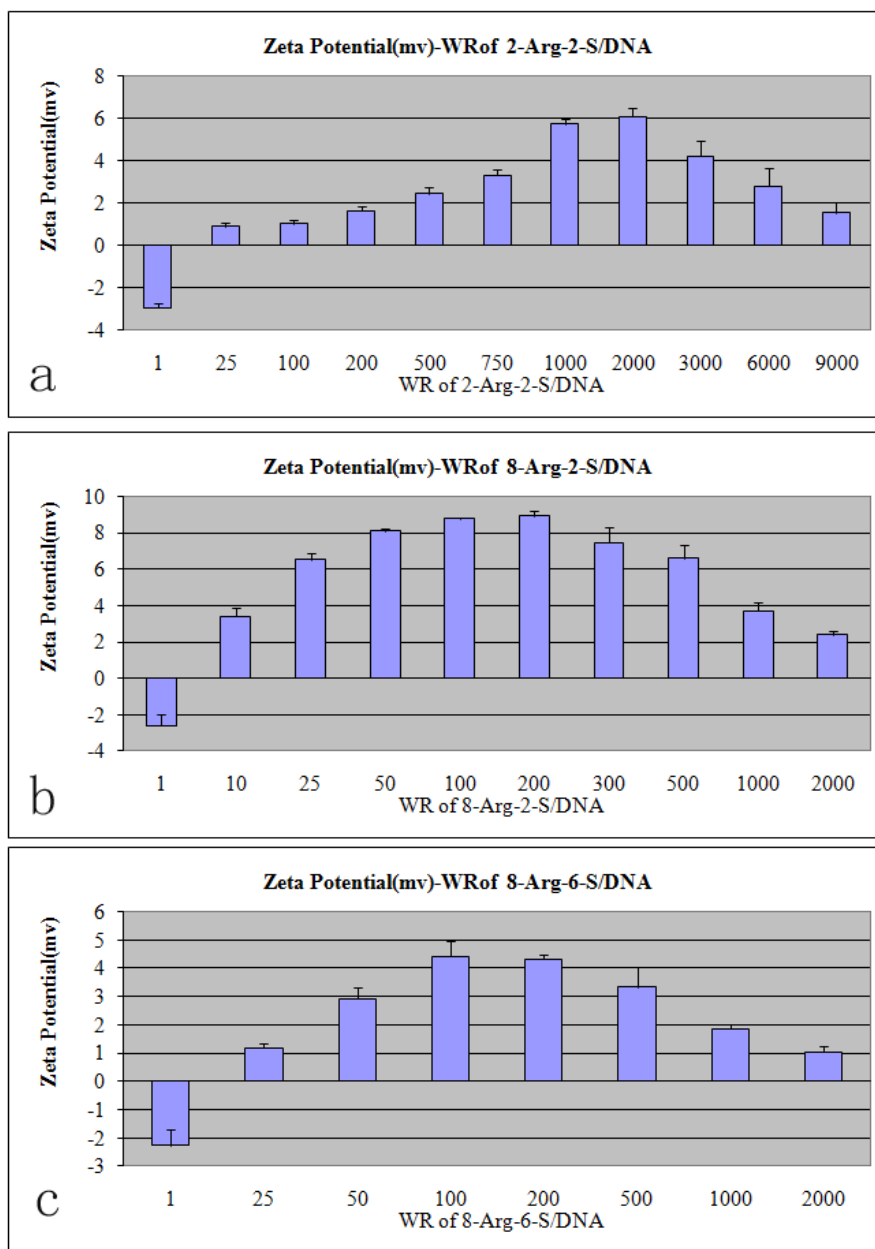


Figure 3.13 Zeta potential measurements of Arg-PEA/DNA complex in a very wide weight ratio (WR) range. Positive value means the complex is positively charged; while negative value means the complex is negatively charged. (a), 2-Arg-2-S/DNA complex; (b), 8-Arg-2-S/DNA complex; (c)8-Arg-6-S/DNA complex

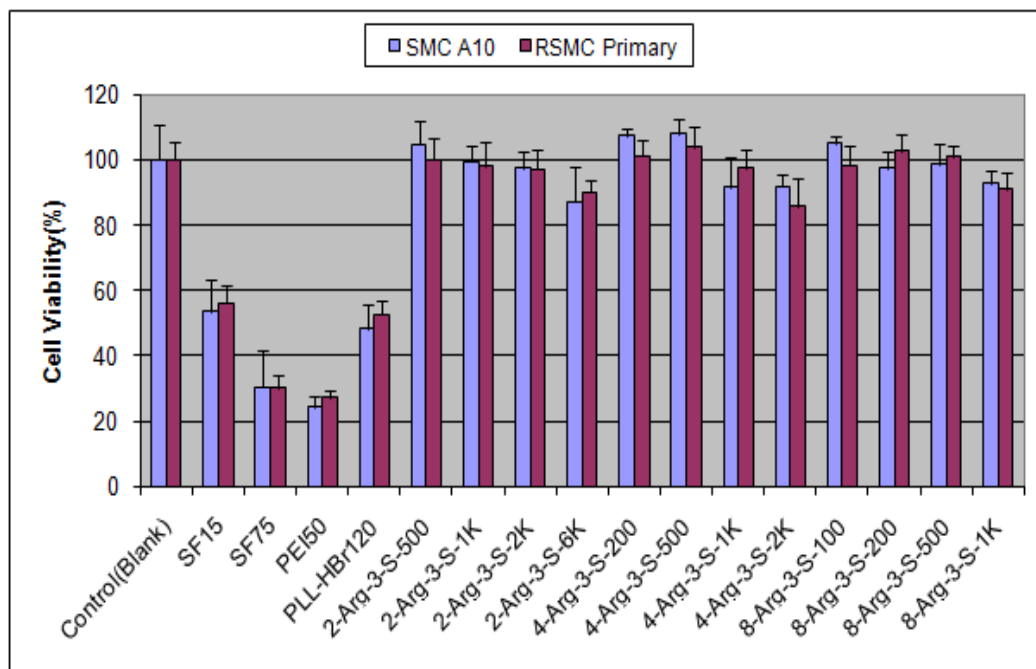


Figure 3.14, Cytotoxicity of Arg-PEA/DNA complexes by MTT assay. The cell viability was normalized against blank control by setting the blank control as 100. Negative control (NC) is cells only without any transfection agent treatment. Various WRs of Arg-PEA to DNA were tested (from 2-Arg-3-S to 8-Arg-3-S). The numerical numbers after the sample label indicate the corresponding WR.

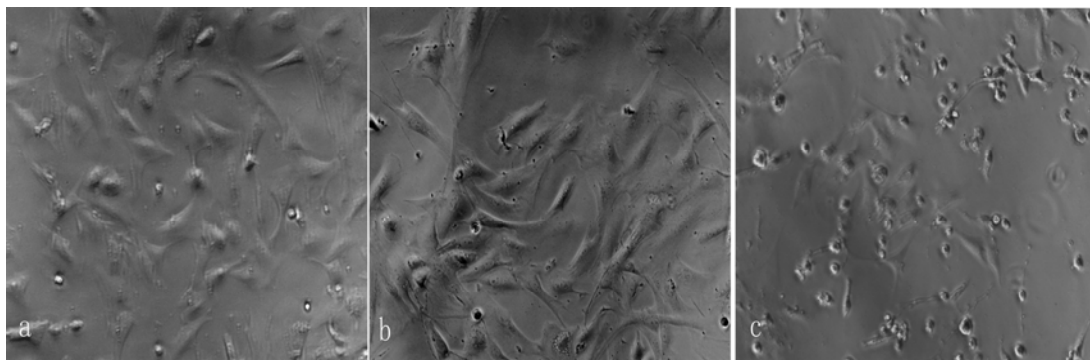


Figure 3.15 RSMC cell morphology (10x, images taken 48 h after treatment): (a) Negative control, no polymer added; (b) cells with 2,000 μg 2-Arg-3-S added for 4 h; (c) cells with 15 μg Superfect® added for 4 h.

3.D.6 Cytotoxicity of Arg-PEA/DNA Complex by MTT Assay

Cytotoxicity of Arg-PEA/DNA complexes was evaluated by MTT assay. The MTT system is a simple, accurate, reproducible means of detecting living cells via mitochondrial dehydrogenase activity. An increase in cell number (cell proliferation) results in an increase in the amount of MTT formazan formed and an increase in UV absorbance. PEI, PLL and Superfect[®] were used as the controls.

All the synthesized Arg-PEAs at different WR of Arg-PEA/DNA were tested by MTT assay as shown in Figure 3.14. The MTT data clearly demonstrated that, at 4 h treatment, all the Arg-PEA/DNA complexes showed very little toxicity to the SMC A10 and RSMC primary cells even at a very large dosage. Although the 3 controls (Superfect[®], PEI and PLL) required much lower dosages than Arg-PEAs to reach optimum transfection efficiency, these 3 controls still showed a significantly higher cytotoxicity than Arg-PEAs. Since Arg-PEA had a lower positive charge density than these 3 control transfection reagents, a larger dose of Arg-PEA was needed to achieve efficient transfection. The statistical data analysis showed that there is no significant difference of any Arg-PEA treatment compared to the control at the p 0.05 level by Dunnet test of planned comparison. So there is no evidence of toxicity of Arg-PEAs.

In addition to MTT assay, the cytotoxicity of Arg-PEA/DNA complexes can also be confirmed by their effect on cell morphology as shown in Figure 3.15. Figure 3.15 showed the images of RSMC primary cells 48h after treatment in different Arg-PEA/DNA complexes for 4 h. It can be seen that the RSMC treated by 2-Arg-2-S displayed normal RSMC morphology, confirming the non-toxic nature of these Arg-PEAs. In contrast, those RSMC primary cells treated by Superfect[®] appeared to be

very unhealthy. So we can conclude that these newly developed Arg-PEAs are non-toxic and very safe to the SMC A10 cells and RSMC primary cell.

3.E Conclusion

In this work, we prepared a series of water soluble, non-toxic and biodegradable L-Arginine based poly (ester amide)s (Arg-PEAs) for both an in-depth study of their capability as a non-toxic and non-viral vector as well as the elucidation of the relationship between polymer structure – biological functions. Through various assays and methods, we confirmed that Arg-PEAs could condense the DNA and form stable complex for a subsequent transfection of aortic smooth muscle cells. Some Arg-PEAs showed better transfection efficiency than Superfect[®], while exhibiting a much lower cytotoxicity. This study revealed a quantitative relationship among Arg-PEA polymer structures, their hydrophilicity and transfection capability. For example, increasing the length of methylene groups in Arg-PEA repeating unit (x or y) decreases the polymer hydrophilicity, and subsequently increases their DNA condensation capability. The Arg-PEA hydrophilicity also had a great impact on both the transfection efficiency and the optimal weight ratio of Arg-PEA to DNA required for the best transfection performance. The zeta potential data of the Arg-PEA/DNA complexes provided a further and strong support for such a relationship of Arg-PEA structure – biological function. This new Arg-PEA family showed a great potential as a better and safer non-viral transfection agent. Their ability to deliver therapeutic DNAs with insignificant cytotoxicity could be further improved by modifying the polymer structure and transfection protocol, such as change polymer molecular weight, the length of methylene group in Arg-PEA and the type of salt other than toluenesulfonic acid salt, etc.

REFERENCE

1. Anderson, W. F., Human Gene-Therapy. *Science (New York, N.Y.)* **1992**, 256, (5058), 808-813.
2. Anderson, W. F., Human gene therapy. *Nature (London)* **1998**, 392, (6679, Suppl.), 25-30.
3. Luo, D.; Saltzman, W. M., Synthetic DNA delivery systems. *Nature biotechnology* **2000**, 18, (1), 33-37.
4. Mulligan, R. C., The Basic Science of Gene-Therapy. *Science (New York, N.Y.)* **1993**, 260, (5110), 926-932.
5. Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S., Design and development of polymers for gene delivery. *Nature Reviews Drug Discovery* **2005**, 4, (7), 581-593.
6. Wolff, J. A.; Lederberg, J., An Early History of Gene-Transfer and Therapy. *Human Gene Therapy* **1994**, 5, (4), 469-480.
7. Wolff, J. A.; Trubetskoy, V. S., The Cambrian period of nonviral gene delivery. *Nature Biotechnology* **1998**, 16, (5), 421-422.
8. Yamanouchi, D.; Wu, J.; Lazar, A. N.; Craig Kent, K.; Chu, C.-C.; Liu, B., Biodegradable arginine-based poly(ester-amide)s as non-viral gene delivery reagents. *Biomaterials* **2008**, 29, (22), 3269-3277.
9. Lynn, D. M.; Anderson, D. G.; Putnam, D.; Langer, R., Accelerated discovery of synthetic transfection vectors: Parallel synthesis and screening of degradable polymer library. *Journal of the American Chemical Society* **2001**, 123, (33), 8155-8156.
10. Mahato, R. I.; Smith, L. C.; Rolland, A., Pharmaceutical perspectives of nonviral gene therapy. In *Advances in Genetics, Vol 41*, 1999; Vol. 41, pp 95-156.
11. Putnam, D., Polymers for gene delivery across length scales. *Nature Materials* **2006**, 5, (6), 439-451.

12. Putnam, D.; Gentry, C. A.; Pack, D. W.; Langer, R., Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, 98, (3), 1200-+.
13. Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P., A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* **1995**, 92, (16), 7297-301.
14. Verma, I. M.; Somia, N., Gene therapy - promises, problems and prospects. *Nature* **1997**, 389, (6648), 239-242.
15. Zauner, W.; Ogris, M.; Wagner, E., Polylysine-based transfection systems utilizing receptor-mediated delivery. *Advanced Drug Delivery Reviews* **1998**, 30, (1-3), 97-113.
16. Katayose, S.; Kataoka, K., Water-Soluble Polyion Complex Associates of DNA and Poly(ethylene glycol)-Poly(L-lysine) Block Copolymer. *Bioconjugate Chemistry* **1997**, 8, (5), 702-707.
17. Wagner, E.; Zatloukal, K.; Cotten, M.; Kirlappos, H.; Mechtler, K.; Curiel, D. T.; Birnstiel, M. L., Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. *Proceedings of the National Academy of Sciences of the United States of America* **1992**, 89, (13), 6099-103.
18. Zhong, Z.; Feijen, J.; Lok, M. C.; Hennink, W. E.; Christensen, L. V.; Yockman, J. W.; Kim, Y.-H.; Kim, S. W., Low molecular weight linear polyethylenimine-b-poly(ethylene glycol)-b-polyethylenimine triblock copolymers: synthesis, characterization, and in vitro gene transfer properties. *Biomacromolecules* **2005**, 6, (6), 3440-3448.

19. Pollard, H.; Remy, J.-S.; Loussouarn, G.; Demolombe, S.; Behr, J.-P.; Escande, D., Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *Journal of Biological Chemistry* **1998**, 273, (13), 7507-7511.
20. Liu, X.; Yang, J. W.; Lynn, D. M., Addition of "Charge-Shifting" Side Chains to Linear Poly(ethyleneimine) Enhances Cell Transfection Efficiency. *Biomacromolecules* **2008**, 9, (7), 2063-2071.
21. Tekade, R. K.; Kumar, P. V.; Jain, N. K., Dendrimers in Oncology: An Expanding Horizon. *Chemical Reviews (Washington, DC, United States)* **2009**, 109, (1), 49-87.
22. Tomalia, D. A.; Naylor, A. M.; Goddard, W. A., Starburst Dendrimers - Molecular-Level Control of Size, Shape, Surface-Chemistry, Topology, and Flexibility from Atoms to Macroscopic Matter. *Angewandte Chemie-International Edition in English* **1990**, 29, (2), 138-175.
23. Wong, K.; Sun, G.; Zhang, X.; Dai, H.; Liu, Y.; He, C.; Leong, K. W., PEI-g-chitosan, a Novel Gene Delivery System with Transfection Efficiency Comparable to Polyethylenimine in Vitro and after Liver Administration in Vivo. *Bioconjugate Chemistry* **2006**, 17, (1), 152-158.
24. Koeping-Hoeggard, M.; Varum, K. M.; Issa, M.; Danielsen, S.; Christensen, B. E.; Stokke, B. T.; Artursson, P., Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers. *Gene Therapy* **2004**, 11, (19), 1441-1452.
25. Wu, J.; Wang, X.; Keum, J. K.; Zhou, H.; Gelfer, M.; Avila-Orta, C.-A.; Pan, H.; Chen, W.; Chiao, S.-M.; Hsiao, B. S.; Chu, B., Water soluble complexes of chitosan-g-MPEG and hyaluronic acid. *Journal of Biomedical Materials Research, Part A* **2007**, 80A, (4), 800-812.

26. Ihm, J. E.; Han, K. O.; Han, I. K.; Ahn, K. D.; Han, D. K.; Cho, C. S., High transfection efficiency of poly(4-vinylimidazole) as a new gene carrier. *Bioconjugate Chemistry* **2003**, 14, (4), 707-708.
27. Midoux, P.; Monsigny, M., Efficient gene transfer by histidylated polylysine pDNA complexes. *Bioconjugate Chemistry* **1999**, 10, (3), 406-411.
28. Pack, D. W.; Putnam, D.; Langer, R., Design of imidazole-containing endosomolytic biopolymers for gene delivery. *Biotechnology and Bioengineering* **2000**, 67, (2), 217-223.
29. Thomas, J. L.; Tirrell, D. A., Polyelectrolyte-Sensitized Phospholipid-Vesicles. *Accounts of Chemical Research* **1992**, 25, (8), 336-342.
30. Thomas, J. L.; Barton, S. W.; Tirrell, D. A., Membrane Solubilization by a Hydrophobic Polyelectrolyte - Surface-Activity and Membrane-Binding. *Biophysical Journal* **1994**, 67, (3), 1101-1106.
31. Lim, Y. B.; Kim, C. H.; Kim, K.; Kim, S. W.; Park, J. S., Development of a safe gene delivery system using biodegradable polymer, poly[alpha-(4-aminobutyl)-L-glycolic acid]. *Journal of the American Chemical Society* **2000**, 122, (27), 6524-6525.
32. Lynn, D. M.; Langer, R., Degradable poly(beta-amino esters): Synthesis, characterization, and self-assembly with plasmid DNA. *Journal of the American Chemical Society* **2000**, 122, (44), 10761-10768.
33. Al-Jamal, K. T.; Ramaswamy, C.; Singh, B.; Florence, A. T., Structures from lysine-based dendrons and dendrimers: Monolayers, dendriplexes, dendrisomes, nanoparticles and micellar aggregates. *Journal of Drug Delivery Science and Technology* **2005**, 15, (1), 11-18.
34. Anderson, D. G.; Akinc, A.; Hossain, N.; Langer, R., Structure/property studies of polymeric gene delivery using a library of poly(beta -amino esters). *Molecular Therapy* **2005**, 11, (3), 426-434.

35. Braun, C. S.; Vetro, J. A.; Tomalia, D. A.; Koe, G. S.; Koe, J. G.; Middaugh, C. R., Structure/function relationships of polyamidoamine/DNA dendrimers as gene delivery vehicles. *Journal of Pharmaceutical Sciences* **2005**, 94, (2), 423-436.
36. Chen, D. J.; Majors, B. S.; Zelikin, A.; Putnam, D., Structure-function relationships of gene delivery vectors in a limited polycation library. *Journal of Controlled Release* **2005**, 103, (1), 273-283.
37. Deshpande, M. C.; Garnett, M. C.; Vamvakaki, M.; Bailey, L.; Armes, S. P.; Stolnik, S., Influence of polymer architecture on the structure of complexes formed by PEG-tertiary amine methacrylate copolymers and phosphorothioate oligonucleotide. *Journal of Controlled Release* **2002**, 81, (1-2), 185-199.
38. Doody, A. M.; Korley, J. N.; Dang, K. P.; Zawaneh, P. N.; Putnam, D., Characterizing the structure/function parameter space of hydrocarbon-conjugated branched polyethylenimine for DNA delivery in vitro. *Journal of Controlled Release* **2006**, 116, (2), 227-237.
39. Fischer, D.; von Harpe, A.; Kunath, K.; Petersen, H.; Li, Y.; Kissel, T., Copolymers of Ethylene Imine and N-(2-Hydroxyethyl)-ethylene Imine as Tools To Study Effects of Polymer Structure on Physicochemical and Biological Properties of DNA Complexes. *Bioconjugate Chemistry* **2002**, 13, (5), 1124-1133.
40. Forrest, M. L.; Koerber, J. T.; Pack, D. W., A Degradable Polyethylenimine Derivative with Low Toxicity for Highly Efficient Gene Delivery. *Bioconjugate Chemistry* **2003**, 14, (5), 934-940.
41. Germershaus, O.; Mao, S.; Sitterberg, J.; Bakowsky, U.; Kissel, T., Gene delivery using chitosan, trimethyl chitosan or polyethyleneglycol-graft-trimethyl chitosan block copolymers: Establishment of structure-activity relationships in vitro. *Journal of Controlled Release* **2008**, 125, (2), 145-154.

42. Green, J. J.; Langer, R.; Anderson, D. G., A Combinatorial Polymer Library Approach Yields Insight into Nonviral Gene Delivery. *Accounts of Chemical Research* **2008**, 41, (6), 749-759.
43. Guo, Y.; Sun, Y.; Li, G.; Xu, Y., Molecular structures of poly(ethylene glycol)-modified nonviral gene delivery polyplexes. *Molecular Pharmaceutics* **2004**, 1, (6), 477-482.
44. Haider, M.; Leung, V.; Ferrari, F.; Crissman, J.; Powell, J.; Cappello, J.; Ghandehari, H., Molecular engineering of silk-elastinlike polymers for matrix-mediated gene delivery: biosynthesis and characterization. *Molecular Pharmaceutics* **2005**, 2, (2), 139-150.
45. Hwang, S. J.; Bellocq, N. C.; Davis, M. E., Effects of Structure of beta - Cyclodextrin-Containing Polymers on Gene Delivery. *Bioconjugate Chemistry* **2001**, 12, (2), 280-290.
46. Jones, N. A.; Hill, I. R. C.; Stolnik, S.; Bignotti, F.; Davis, S. S.; Garnett, M. C., Polymer chemical structure is a key determinant of physicochemical and colloidal properties of polymer-DNA complexes for gene delivery. *Biochimica et Biophysica Acta, Gene Structure and Expression* **2000**, 1517, (1), 1-18.
47. Laga, R.; Konak, C.; Subr, V.; Ulbrich, K., New, Hydrophilic, HPMA-Based Polymers for Bioresponsive Shielding of Gene-Delivery Vectors. *Macromolecular Chemistry and Physics* **2009**, 210, (13-14), 1138-1148.
48. Lee, C.-C.; Liu, Y.; Reineke, T. M., General Structure-Activity Relationship for Poly(glycoamidoamine)s: The Effect of Amine Density on Cytotoxicity and DNA Delivery Efficiency. *Bioconjugate Chemistry* **2008**, 19, (2), 428-440.
49. Lobo, B. A.; Vetro, J. A.; Suich, D. M.; Zuckermann, R. N.; Middaugh, C. R., Structure/function analysis of peptoid/lipitoid: DNA complexes. *Journal of Pharmaceutical Sciences* **2003**, 92, (9), 1905-1918.

50. Mannisto, M.; Vanderkerken, S.; Toncheva, V.; Elomaa, M.; Ruponen, M.; Schacht, E.; Urtti, A., Structure-activity relationships of poly(L-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. *Journal of Controlled Release* **2002**, 83, (1), 169-182.
51. Pietersz, G. A.; Tang, C.-K.; Apostolopoulos, V., Structure and design of polycationic carriers for gene delivery. *Mini-Reviews in Medicinal Chemistry* **2006**, 6, (12), 1285-1298.
52. Reineke, T. M.; Davis, M. E., Structure-property investigation of trehalose and beta -cyclodextrin-based polycations for gene delivery. *PMSE Preprints* **2003**, 88, 224-225.
53. Zhu, L.; Lu, Y.; Miller, D. D.; Mahato, R. I., Structural and Formulation Factors Influencing Pyridinium Lipid-Based Gene Transfer. *Bioconjugate Chemistry* **2008**, 19, (12), 2499-2512.
54. Banerjee, P.; Weissleder, R.; Bogdanov, A., Jr., Linear Polyethyleneimine Grafted to a Hyperbranched Poly(ethylene glycol)-like Core: A Copolymer for Gene Delivery. *Bioconjugate Chemistry* **2006**, 17, (1), 125-131.
55. Choi, J. S.; Lee, E. J.; Choi, Y. H.; Jeong, Y. J.; Park, J. S., Poly(ethylene glycol)-block-poly(L-lysine) Dendrimer: Novel Linear Polymer/Dendrimer Block Copolymer Forming a Spherical Water-Soluble Polyionic Complex with DNA. *Bioconjugate Chemistry* **1999**, 10, (1), 62-65.
56. Cheng, H.; Zhu, J.-L.; Zeng, X.; Jing, Y.; Zhang, X.-Z.; Zhuo, R.-X., Targeted Gene Delivery Mediated by Folate-polyethylenimine-block-poly(ethylene glycol) with Receptor Selectivity. *Bioconjugate Chemistry* **2009**, 20, (3), 481-487.
57. Burke, R. S.; Pun, S. H., Extracellular Barriers to in Vivo PEI and PEGylated PEI Polyplex-Mediated Gene Delivery to the Liver. *Bioconjugate Chemistry* **2008**, 19, (3), 693-704.

58. Fukushima, S.; Miyata, K.; Nishiyama, N.; Kanayama, N.; Yamasaki, Y.; Kataoka, K., PEGylated Polyplex Micelles from Triblock Cationomers with Spatially Ordered Layering of Condensed pDNA and Buffering Units for Enhanced Intracellular Gene Delivery. *Journal of the American Chemical Society* **2005**, 127, (9), 2810-2811.
59. Wong, S. Y.; Sood, N.; Putnam, D., Combinatorial Evaluation of Cations, pH-sensitive and Hydrophobic Moieties for Polymeric Vector Design. *Molecular Therapy* **2009**, 17, (3), 480-490.
60. Guo, K.; Chu, C. C., Synthesis, Characterization, and Biodegradation of Novel Poly(ether ester amide)s Based on L-Phenylalanine and Oligoethylene Glycol. *Biomacromolecules* **2007**, 8, (9), 2851-2861.
61. Guo, K.; Chu, C. C., Synthesis, characterization, and biodegradation of copolymers of unsaturated and saturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2007**, 45, (9), 1595-1606.
62. Chu, C.-C., Biodegradable polymeric biomaterials: an updated overview. *Biomaterials* **2007**, 6/1-6/22.
63. Guo, K.; Chu, C. C.; Chkhaidze, E.; Katsarava, R., Synthesis and characterization of novel biodegradable unsaturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2005**, 43, (7), 1463-1477.
64. Katsarava, R.; Beridze, V.; Arabuli, N.; Kharadze, D.; Chu, C. C.; Won, C. Y., Amino acid-based bioanalogous polymers. synthesis, and study of regular poly(ester amide)s based on bis(alpha -amino acid) alpha ,w-alkylene diesters, and aliphatic dicarboxylic acids. *Journal of Polymer Science, Part A: Polymer Chemistry* **1999**, 37, (4), 391-407.
65. Deng, M.; Wu, J.; Reinhart-King, C. A.; Chu, C.-C., Synthesis and Characterization of Biodegradable Poly(ester amide)s with Pendant Amine Functional

Groups and in Vitro Cellular Response. *Biomacromolecules* **2009**, 10, (11), 3037-3047.

66. Futaki, S.; Ohashi, W.; Suzuki, T.; Niwa, M.; Tanaka, S.; Ueda, K.; Harashima, H.; Sugiura, Y., Stearylated arginine-rich peptides: A new class of transfection systems. *Bioconjugate Chemistry* **2001**, 12, (6), 1005-1011.

67. Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B., Polyarginine enters cells more efficiently than other polycationic homopolymers. *Journal of Peptide Research* **2000**, 56, (5), 318-325.

68. Cheng, N.; Liu, W.; Cao, Z.; Ji, W.; Liang, D.; Guo, G.; Zhang, J., A study of thermoresponsive poly(N-isopropylacrylamide)/polyarginine bioconjugate non-viral transgene vectors. *Biomaterials* **2006**, 27, (28), 4984-92.

69. Holowka Eric, P.; Sun Victor, Z.; Kamei Daniel, T.; Deming Timothy, J., Polyarginine segments in block copolypeptides drive both vesicular assembly and intracellular delivery. *Nat Mater* **2007**, 6, (1), 52-7.

70. Liu, B.; Itoh, H.; Louie, O.; Kubota, K.; Kent, K. C., The signaling protein Rho is necessary for vascular smooth muscle migration and survival but not for proliferation. *Surgery* **2002**, 132, (2), 317-25.

71. Tsai, S.; Hollenbeck, S. T.; Ryer, E. J.; Edlin, R.; Yamanouchi, D.; Kundi, R.; Wang, C.; Liu, B.; Kent, K. C., TGF-beta through Smad3 signaling stimulates vascular smooth muscle cell proliferation and neointimal formation. *American Journal of Physiology* **2009**, 297, (2, Pt. 2), H540-H549.

72. Zhang, F.; Kent, K. C.; Yamanouchi, D.; Zhang, Y.; Kato, K.; Tsai, S.; Nowygrod, R.; Schmidt Ann, M.; Liu, B., Anti-receptor for advanced glycation end products therapies as novel treatment for abdominal aortic aneurysm. *Annals of surgery* **2009**, 250, (3), 416-23.

73. Brito, L.; Little, S.; Langer, R.; Amiji, M., Poly(beta -amino ester) and Cationic Phospholipid-Based Lipopolyplexes for Gene Delivery and Transfection in Human Aortic Endothelial and Smooth Muscle Cells. *Biomacromolecules* **2008**, 9, (4), 1179-1187.
74. Perlstein, I.; Connolly, J. M.; Cui, X.; Song, C.; Li, Q.; Jones, P. L.; Lu, Z.; DeFelice, S.; Klugherz, B.; Wilensky, R.; Levy, R. J., DNA delivery from an intravascular stent with a denatured collagen-poly(lactic-polyglycolic acid)-controlled release coating: mechanisms of enhanced transfection. *Gene Therapy* **2003**, 10, (17), 1420-1428.
75. Vera, M.; Almontassir, A.; Rodriguez-Galan, A.; Puiggali, J., Synthesis and Characterization of a New Degradable Poly(ester amide) Derived from 6-Amino-1-hexanol and Glutaric Acid. *Macromolecules* **2003**, 36, (26), 9784-9796.
76. Vera, M.; Franco, L.; Puiggali, J., Synthesis of poly(ester amide)s with lateral groups from a bulk polycondensation reaction with formation of sodium chloride salts. *Journal of Polymer Science, Part A: Polymer Chemistry* **2007**, 46, (2), 661-667.
77. Layman, J. M.; Borgerding, E. M.; Williams, S. R.; Appelhans, D.; Voit, B.; Long, T. E., Elucidating structure-property relationships in polycation-mediated gene delivery. *PMSE Preprints* **2008**, 98, 303-304.
78. Zelikin, A. N.; Putnam, D.; Shastri, P.; Langer, R.; Izumrudov, V. A., Aliphatic Ionenics as Gene Delivery Agents: Elucidation of Structure-Function Relationship through Modification of Charge Density and Polymer Length. *Bioconjugate Chemistry* **2002**, 13, (3), 548-553.

CHAPTER 4

ADVANCED GENERATION OF AMINO ACID-BASED POLY (ESTER AMIDE)S
AS NON-VIRAL GENE DELIVERY VECTOR FOR PRIMARY AND STEM
CELLS AND STRUCTURE-FUNCTION STUDY

Jun Wu,¹ Dai Yamanouchi,³ Bo Liu,³ C.C. Chu^{1,2}

¹Department of Biomedical Engineering, Cornell University, Ithaca, NY 14853-4401,
USA

²Department of Fiber Science and Apparel Design, Cornell University, Ithaca, NY
14853-4401, USA

³Department of Surgery, University of Wisconsin, Madison, WI 53705-2275, USA

4.A Abstract

The goals of this study are to evaluate a family of water soluble, cationic L-arginine based poly (ether ester amide)s (Arg-PEEAs) as non-viral gene delivery vectors for cell lines, primary cells and stem cells. These new biodegradable polymers consist of 3 non-toxic building blocks: L-arginine, oligoethylene glycols, and dicarboxylic acids. The Arg-PEEAs were prepared by the solution polycondensation reaction of *p*-toluenesulfonic acid salt of L-arginine diester from oligoethylene glycol and di-*p*-nitrophenyl esters of dicarboxylic acids. The optimal conditions of the synthesis of both monomers and polymers were investigated, and the newly prepared monomers and polymers were chemically characterized. Arg-PEEAs were found to have very good solubility in water and many other polar solvents. It was found that the introduction of oligoethylene glycols into the polymer backbone increased the polymer water solubility and decreased the glass transition temperature (T_g) of the polymers. The relationship between Arg-PEEAs backbone structure and their gene transfection efficiency and Arg-PEEA/DNA cytotoxicity were studied through various biological assays, such as gel retardation assay, GFP assay, luciferase assay and MTT assay. The transfection results obtained from luciferase and GFP assays for many types of cell lines, primary cells and stem cells showed that some Arg-PEEAs had higher transfection efficiency than the commercial transfection reagent Lipofectamine2000[®], but at a much lower cytotoxicity. Zeta potential and particle size tests provided more details of the Arg-PEEA/DNA complex properties.

4.B Introduction

Gene therapy can be defined as the treatment of human disease by transferring the genetic material into specific cells of patients. During the past several decades, with the fast growing molecular biology techniques, gene therapy technology has been developed rapidly¹⁻⁶. Since the first treatment of patients with gene transfer techniques under the approved FDA protocols in 1990, more than 1000 gene therapy clinical trials have been approved worldwide. However, the successful rate of gene therapy is not very encouraging¹⁻⁶. Based on the reported gene trial results, one of the key limitations is that there have not been safe, efficient and controllable methods for gene delivery³⁻⁸.

Gene delivery can be mainly accomplished by either virus or non-viral transfer methods. The viruses utilized for gene therapy could be divided into retroviruses and adenoviruses². The advantages and disadvantages of viral delivery have been well documented². Based on the aspect of the clinical safety, the non-viral gene delivery method appears to be the most promising approach. For the reported non-viral gene delivery vectors, most of them can be divided into the following 4 broad categories: water soluble cationic polymers, lipids, dendrimers and nanoparticles. Among them, the water soluble synthetic and natural polycations have attracted the most attentions^{7, 9-11}. A large number of cationic polymers have been tested for gene delivery. Among them, poly-L-lysine (PLL)¹¹ and polyethylenimine (PEI)⁹ have been intensively studied because of their strong interaction with the plasmid DNA, resulting the formation of a compact polymer/DNA complex. Other synthetic and natural polycations developed as non-viral vectors includes polyamidoamine dendrimers^{12, 13} and chitosan^{14, 15}, imidazole-containing polymers with proton-sponge effect^{16, 17},

membrane-disruptive peptides and polymers like polyethylacrylic acid (PEAA)^{18, 19}, poly [α -(4-aminobutyl)-L-glycolic acid] (PAGA)²⁰, and poly (amino acid) based materials²¹. However, most of them could not achieve both high transfection efficiency and low toxicity simultaneously.

Recently, many important reported studies focused on how the polycation's property/structure affect the transfection efficiency or decrease the cytotoxicity of polycations²²⁻³¹. Putnam et al reported how to improve the transfection efficiency by balancing the side-chain termini of polylysine conjugated with imidazole groups⁷. Anderson et al prepared more than 500 poly (β -amino esters) (PBAEs) with 70 primary structures to study the polymer structure/properties relationship for gene delivery²². They found that certain polymer structural characteristics are important for effective gene delivery and the best PBAEs are linear polymers of ~ 10 kDa that contain hydroxyl side chains and primary amine end groups²². Wong et al did an interesting research to explore the relationship between gene delivery efficiency and polymer pendant groups' hydrophilic/hydrophobic properties³². In order to have a better understanding of the relationship of polycation structure-function, we have reported a simple, but precise model, L- arginine based poly (ester amide) (Arg-PEAs, Figure 4.1) to quantitatively study how the polymer structure affect their gene delivery performance⁶.

This Arg-PEA system has the following unique advantages: (1) The diacid (x) and diol (y) parts of the PEA repeating unit could be precisely controlled and adjusted by selecting different monomers; (2) L-arginine carries a positive charge at physiological pH due to the guanidino group, a very strong basic group with an isoelectric point of 10.96 and pKa about 12.5, which could have a strong potential to condense negatively

charged nuclear acids; (3) Arg-based polymers have been shown to enter cells efficiently. Futaki et al. and Mitchell et al have reported in their in vitro studies that the poly (L-arginine) can enter the cells more efficiently than other polycationic homopolymers^{33, 34}, however, high molecular weight poly (L-arginine) was found to be very toxic to cells³⁵. Our previous results⁶ have shown that the 1st generation Arg-PEAs using aliphatic alcohols showed excellent cell membrane penetrating capability, high transfection efficiency and low cytotoxicity to cell lines, such as rat smooth muscle cell (SMC) A10 cell line, when compared with commercial transfection agents: Superfect[®] and Lipofectamine2000[®]. However, those 1st generation Arg-PEAs could not transfect the primary and stem cells with high efficiency.

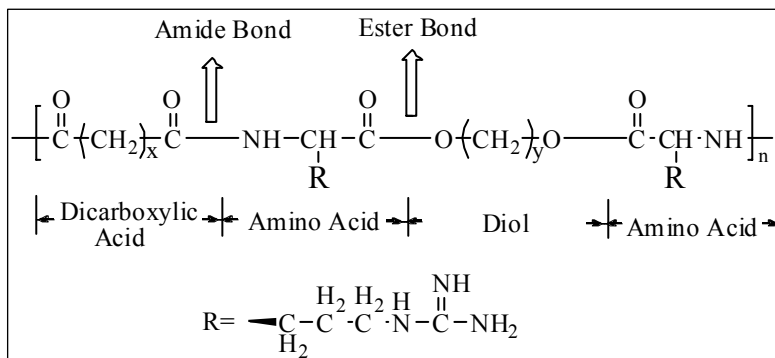


Figure 4.1 General Chemical Structure of Arginine Based Poly (ester amide)

Due to the limitation of the cell types that can be transfected properly by the 1st generation Arg-PEA, a newer generation of arginine based PEA is needed. According to our unpublished results, the Arg-PEAs having double bonds in the polymer backbone showed significantly higher T_g and reduced transfection efficiency than those having saturated moieties.

Therefore, we have the following hypothesis: Flexible Arg-based poly (ester amide)s chains could show increased transfection efficiency because the more flexible Arg-PEA polymers could wrap around the relatively rigid triple helix plasma DNA better for an improved DNA condensation. For this study, oligoethylene glycols of various molecular weights were selected as the candidates for the diol part of the Arg-PEAs because of their very flexible backbone structure. As we know, PEG chain had been widely conjugated to the polycation's backbone or side chain to form an amphiphilic structure of the polycations^{15, 29-31, 36, 37}. In most of those published studies, the conjugation of PEG segments could reduce the cytotoxicity of polymer/DNA complex and improve the transfection efficiency. These PEG segments used for conjugation had molecular weights ranging from 600 to 5,000^{15, 29-31, 36, 37}. There are very few publications discussed the introduction of short chain PEG, oligoethylene glycol (molecular weight below 600, such as diethylene glycol), to the polymer repeating units for gene transfection study and other biomedical applications^{25, 38}.

In this paper, we advanced our 1st generation Arg-PEA study for gene transfection further by introducing oligoethylene glycols into Arg-PEA polymer backbone to have a more flexible chain structure for exploring a better understanding of the relationship between the Arg-PEEA structure and their biological functions.

In this work, a series of Arg-PEEAs (Figure 4.2) having different methylene (x)/ethylene glycol (y) chain length (x=2, 4, 8; y=2, 3, 4, 6, 12) in the repeating unit were prepared to test our hypothesis that the oligoethylene glycol based Arg-PEEAs can improve the transfection efficiency. These Arg-PEEAs would have a wide range of properties for a systematic evaluation of the relationship between chemical structure of

the polymers, their properties and transfection efficiency. The findings could help achieve a better understanding of the structure-function relationship of this new family of water soluble cationic biodegradable Arg-based cationic polymers.

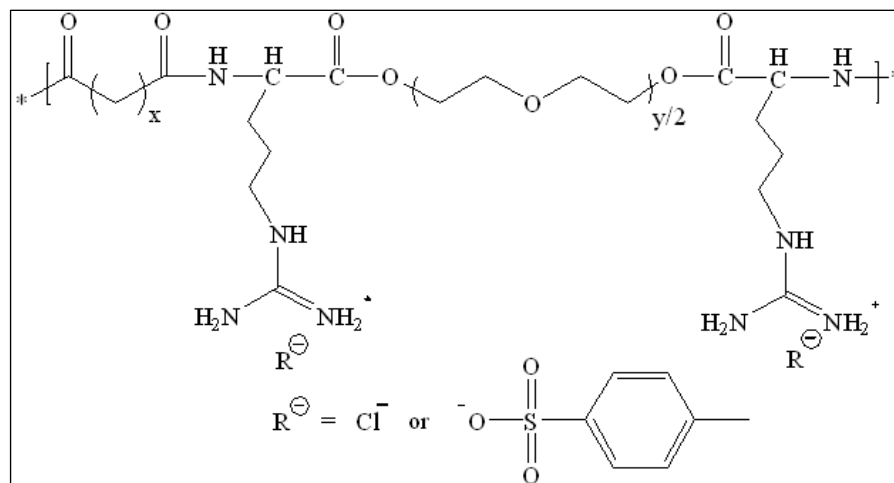


Figure 4.2 Chemical structure of Arg-PEEAs: x-Arg-yEG-z, where x is the number of methylene groups between two adjacent amide bonds, and y is the number of ethylene glycol groups between two adjacent ester groups. z stands for salt type (toluenesulfonic acid salt, Tos or chlorine salt, Cl).

4.C Experimental

4.C.1 Materials

L-Arginine, L-Arginine hydrochloride, *p*-toluenesulfonic acid monohydrate, succinyl chloride, adipoyl chloride, sebacoyl chloride, di-ethylene glycol (DEG), tri-ethylene glycol (TEG), tetra-ethylene glycol (TTEG), poly (ethylene glycol) ($M_n=300$), poly (ethylene glycol) ($M_n=600$), triethylamine and *p*-nitrophenol were all purchased from Alfa Aesar (Ward Hill, MA) and used without further purification.

Organic solvents like methanol, toluene, ethyl acetate, acetone, 2-propanol and dimethyl sulfoxide (DMSO) were purchased from VWR Scientific (West Chester, PA) and were purified by standard methods before use. Other chemicals and reagents if not otherwise specified were purchased from Sigma (St. Louis, MO).

Linear polyethylenimine (PEI) with a weight average molecular weight (M_w) of 25,000, ethidium bromide, MTT, Dulbecco's phosphate-buffered saline (PBS, pH 7.4), TAE, HEPES and other buffers were purchased from Sigma (St. Louis, MO). Dulbecco's modified eagle medium (DMEM), penicillin–streptomycin (PS, 100 U/mL), trypsin–EDTA (TE, 0.5 % trypsin, 5.3 mM EDTA tetra-sodium), fetal bovine serum (FBS) were obtained from Gibco BRL (Rockville, MD). Cell lines (rat SMC A10, BAEC endothelial cells), rat primary smooth muscle cells (RSMC), human umbilical vein endothelial cells (HUVEC) and rat Mesenchymal stem cells, MSC) and rat bone marrow cells (BM)) were obtained from American Type Culture Collection (ATCC, Manassas, VA) or Professor Bo Liu's lab at Surgery Department of Wisconsin University. DNA size marker N3014 was purchased from New England Lab (Woburn, MA). A Qiagen endotoxin-free plasmid Maxi kit was purchased from Qiagen (Valencia, CA). Lipofectamine2000[®] was purchased from Invitrogen (Carlsbad, CA). Promega Luciferase Assay Kit containing luciferase cell culture lyses reagent and luciferase substrates were obtained from Promega (Madison, WI).

4.C.2 Synthesis of Monomers and Polymers

The general scheme of Arg-PEEA synthesis was divided into the following three major steps: 1): the preparation of di-*p*-nitrophenyl ester of dicarboxylic acids (**I**) (Figure 4.3); 2): the synthesis of *p*-toluenesulfonic acid salt of L-arginine diester (**II**)

from di- ethylene glycol, tri-ethylene glycol, and tetra-ethylene glycol, PEG300 and PEG600 (the preparation of tetra-*p*- toluenesulfonic acid salts of bis-L-Arginine esters (**IIa**) (Figure 4.4) and the preparation of di-*p*- toluenesulfonic acid di-hydrochloride acid salts of bis-L-Arginine esters (**IIb**)) (Figure 4.5); and 3): the synthesis of Arg-PEEAs (**III**) (Figure 4.2) via the solution polycondensation of monomers (**I**) and (**IIa** or **IIb**).

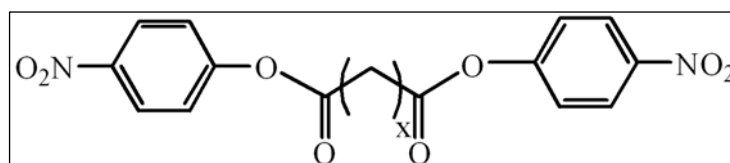


Figure 4.3 Monomer **I**: di-*p*-nitrophenyl ester of dicarboxylic acids

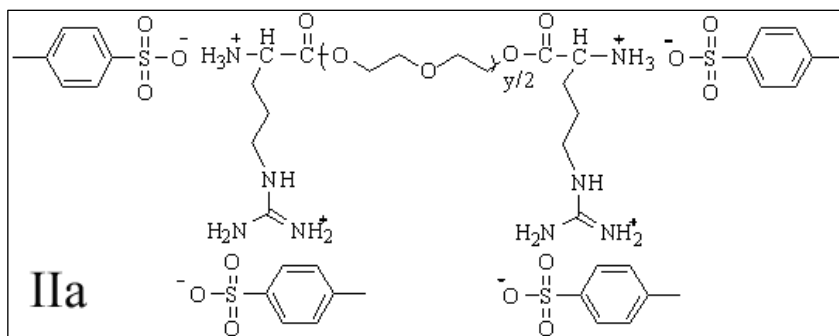


Figure 4.4 Monomer **IIa**: tetra-*p*- toluenesulfonic acid salts of bis-L-Arginine esters

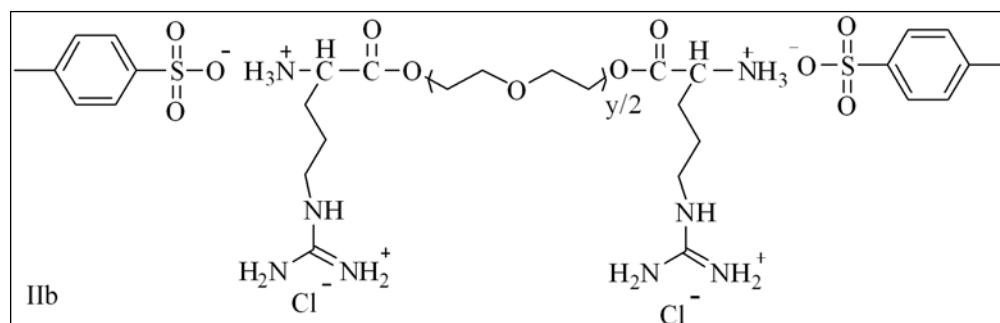


Figure 4.5 Monomer **IIb**: di-*p*-toluenesulfonic acid di-hydrochloride acid salts of bis-L-Arginine esters

Di-*p*-nitrophenyl esters of dicarboxylic acids (Monomer **I**) were prepared by reacting dicarboxylic acyl chloride varying in methylene length (*x*) with *p*-nitrophenol based on our previously reported studies³⁹. Three Monomers **I** were prepared: di-*p*-Nitrophenyl Succinate (**NSu** with *x*=2); di-*p*-Nitrophenyl Adipate (**NA** with *x*=4); di-*p*-Nitrophenyl Sebacate (**NS** with *x*=8). *x* indicates the numbers of methylene group in the diacid.

L-arginine is used for the preparation of tetra-*p*-toluenesulfonic acid salts of bis-L-Arginine esters (**IIa**). Because of the strong positive charge characteristic of L-arginine, the amounts of *p*-toluenesulfonic acid used for the synthesis of *p*-toluenesulfonic acid salt of L-arginine diester was doubled when compared with the prior synthesis of *p*-toluenesulfonic acid salt of non-ionic hydrophobic amino acids diesters. The need to double the amounts of *p*-toluenesulfonic acid in the synthesis of Monomer **IIa** is because of the preferential consumption of the *p*-toluenesulfonic acid by the strong basic guanidine group on L-arginine side chain, and additional *p*-toluenesulfonic acid was needed to convert the free amine groups at the two ends of

the monomer **IIa** into the *p*-toluenesulfonic acid salts for subsequent solution polycondensation with monomer **I**.

An example of the synthesis of monomer **IIa** is given here: L-arginine (0.04 mol) and di-ethylene glycol (0.02 mol) were directly mixed in a three neck round bottom flask with toluene (b.p. 110 °C) (400 mL) with the presence of *p*-toluenesulfonic acid monohydrate (0.082 mol). The solid-liquid reaction mixture was heated to 130 °C and reflux for 24 hr after 2.16 mL (0.12 mol) of water was generated. The reaction mixture (viscous solid) was then cooled to room temperature. Toluene was decanted. The dried reacted mixture was finally purified by repeated precipitation in 2-propanol for three times. 2-propanol was decanted, and then the white sticky mass was dried in vacuum. Five monomers (**IIa**) were made in this study (Table 4.1): **Arg-2E-S**, **Arg-3E-S**, **Arg-4E-S**, **Arg-6E-S**, and **Arg-12E-S**. 2E, 3E, 4E stand for the di-ethylene glycol, tri-ethylene glycol and tetra-ethylene glycol, respectively; 6E stands for PEG300 because the number of ethylene glycols of PEG300 is around 6-7; 12E stands for PEG600 because the number of ethylene glycols of PEG600 is around 12-13. The first 3 monomers (**Arg-2E-S**, **Arg-3E-S**, and **Arg-4E-S**) are white solid powder and the last two are transparent or yellow viscous solid. All of them are obtained in high yields (80~90 %).

L-Arginine hydrochloride is used for the preparation of di-*p*-toluenesulfonic acid di-hydrochloride acid salts of bis-L-arginine esters (**IIb**). Since the basic guanidine group on arginine side chain has formed the salt with hydrochloride acid, the amount of *p*-toluenesulfonic acid used for the synthesis of di-*p*-toluenesulfonic acid di-hydrochloride acid salts of bis-L-arginine esters (**IIb**) was the same as the prior synthesis of *p*-toluenesulfonic acid salt of non-ionic hydrophobic amino acids diesters.

Five monomers (**IIb**) were made in this study (Table 4.1): **Arg-2E-Cl**, **Arg-3E-Cl**, **Arg-4E-Cl**, **Arg-6E-Cl**, and **Arg-12E-Cl**. The definitions of 2E, 3E, 4E, 6E and 12E are same as the monomer **IIa** described above. The first 3 monomers are white solid powder and the last two are transparent or yellow viscous solid. All of them are obtained in high yields (80~90%). All the prepared monomers (**IIa** and **IIb**) are listed in Table 4.1 and labeled as Arg-yEG-z, where y is the number of ethylene glycol units in diols and z is the salt type (S for *p*-toluenesulfonic acid salt or Cl for hydrochloride salt).

Arg-PEEAs (Figure 4.2) were prepared by a solution polycondensation of the monomers (**I**) and (**IIa or IIb**) of different combinations in DMSO solvent, and the synthesized Arg-PEEAs are listed in Table 4.2. An example of the synthesis of 2-Arg-2E-S via solution polycondensation is given here. Monomers **NSu** (1.0 mmol) and **Arg-2E-S** (1.0 mmol) in 1.5 mL of dry DMSO were mixed well by vortexing. The mixture solution was heated up with stirring to obtain a uniformed solution mixture. Triethylamine (0.31 mL, 2.2 mmol) was added drop by drop to the mixture while heating up to 75 °C with vigorous stirring until a complete dissolution of the monomers. The solution color turned into yellow after several minutes. The reaction vial was then kept for 48 hrs at 75 °C in a thermostat oven without stirring. The 2-Arg-2E-S polymer in the reaction solution was precipitated out by adding cold ethyl acetate, decanted, dried, re-dissolved in methanol and re-precipitated in cold ethyl acetate for further purifications. Repeat the purification for 2 times before drying *in vacuo* at room temperature. The prepared Arg-PEEAs are white solid powder (for EG with number 2, 3 and 4) or transparent/yellow viscous solid (for EG with number 6 and 12). All of them are obtained in high yields (80~90 %).

All the Arg-PEEAs are labeled as x-Arg-yEG-z, where x and y are the number of methylene groups in diacids and ethylene glycol units in diols, respectively, and z is the salt type (S for *p*-toluenesulfonic acid salt or Cl for hydrochloride salt).

4.C.3 Characterizations

The physicochemical properties of the prepared monomers and polymers were characterized by various standard methods. For Fourier transform infrared (FTIR) characterization, the samples were ground into powders and mixed with KBr at a sample/KBr ratio of 1:10 (w/w). FTIR spectra were then obtained with a Perkin-Elmer (Madison, WI) Nicolet Magana 560 FTIR spectrometer with Omnic software for data acquisition and analysis. ¹H-NMR spectra were recorded with a Varian Unity Inova 400-MHz spectrometer (Palo Alto, CA). Deuterated water (D₂O-*d*2; Cambridge Isotope Laboratories, Andover, MA) with tetramethylsilane as an internal standard or deuterated dimethyl sulfoxide (DMSO-*d*6; Cambridge Isotope Laboratories) was used as the solvent. MestReNova software was used for the data analysis. The thermal properties of the synthesized Arg-PEEAs were characterized with a DSC 2920 (TA Instruments, New Castle, DE). The measurements were carried out from -20 to 200 °C at a scanning rate of 10 °C/min and at a nitrogen gas flow rate of 25 mL/min. TA Universal Analysis software was used for thermal data analysis. The solubility of Arg-PEEAs in common organic solvents at room temperature was assessed by using 2.0 mg/mL as a solubility criterion to determine whether Arg-PEEA polymer is soluble or not in a solvent. The quantitative solubility of Arg-PEEAs in distilled water at room temperature was measured by adding distilled water drop by drop until a clear solution was obtained. The reduced viscosity (η_{red}) of the polymers synthesized was determined

by a Cannon-Ubbelohde viscometer in DMSO solution at a concentration of 0.25 g/dL at 25 °C.

4.C.4 Electrophoresis Assay

The Arg-PEEA/DNA complexes for agarose gel electrophoresis assay were prepared by adding the DNA marker (N3014 DNA maker) solution into the Arg-PEEA aqueous solutions (in 1X PBS buffer). After mixing the two solutions together, it was immediately vortex for 2-3 seconds, and then equilibrated at an ambient condition for 30 minutes. Arg-PEEA/DNA complexes were analyzed by electrophoresis in a 1 % agarose gel stained with ethidium bromide (10 µg/mL) with TAE buffer at 100 V for 90 min. Total injection volume was 15 µL which consisted of 2 µL blue dye solution, 2 µL DNA marker solution (500 µg/mL), several µL of the Arg-PEEA polymer PBS solution and several µL of pure PBS buffer solution. The Arg-PEEA solutions must be made freshly or stored at 4 °C before use. The amount of DNA was fixed at 1µg per test. After mixing all the solutions, the final system was shaken or centrifuged heavily for several seconds. The N3014 DNA marker solution without Arg-PEEA was used as a blank control. The N3014 DNA marker was visualized by an UV illumination (FOTO/UV 300 Transilluminator). The migration of DNA from the Arg-PEEA/DNA complex was recorded by a digital camera (Panasonic WV-BP330).

Table 4.1 List of prepared *p*-toluenesulfonic acid salt of L-arginine diester from oligoethylene glycols

Monomer	y	Naming
Arg-2E-S	2	tetra- <i>p</i> -toluenesulfonic acid salt of bis (L-arginine) diesters of diethylene glycol
Arg-3E-S	3	tetra- <i>p</i> -toluenesulfonic acid salt of bis (L-arginine) diesters of triethylene glycol
Arg-4E-S	4	tetra- <i>p</i> -toluenesulfonic acid salt of bis (L-arginine) diesters of tetraethylene glycol
Arg-6E-S	6	tetra- <i>p</i> -toluenesulfonic acid salt of bis (L-arginine) diesters of PEG300
Arg-12E-S	12	tetra- <i>p</i> -toluenesulfonic acid salt of bis (L-arginine) diesters of PEG600
Arg-2E-Cl	2	di- <i>p</i> -toluenesulfonic acid di-hydrochloride acid salt of bis (L-arginine) diesters of diethylene glycol
Arg-3E-Cl	3	di- <i>p</i> -toluenesulfonic acid di-hydrochloride acid salt of bis (L-arginine) diesters of triethylene glycol
Arg-4E-Cl	4	di- <i>p</i> -toluenesulfonic acid di-hydrochloride acid salt of bis (L-arginine) diesters of tetraethylene glycol
Arg-6E-Cl	6	di- <i>p</i> -toluenesulfonic acid di-hydrochloride acid salt of bis (L-arginine) diesters of PEG300
Arg-12E-Cl	12	di- <i>p</i> -toluenesulfonic acid di-hydrochloride acid salt of bis (L-arginine) diesters of PEG600

Table 4.2 Arg-PEEAs (x-Arg-yEG-z) prepared by different combinations of diacids and oligoethylene glycol building blocks

	NSu	NA	NS
Arg-2EG-S	2-Arg-2EG-S	4-Arg-2EG-S	8-Arg-2EG-S
Arg-3EG-S	2-Arg-3EG-S	4-Arg-3EG-S	8-Arg-3EG-S
Arg-4EG-S	2-Arg-4EG-S	4-Arg-4EG-S	8-Arg-4EG-S
Arg-6EG-S	2-Arg-6EG-S	4-Arg-6EG-S	8-Arg-6EG-S
Arg-12EG-S	2-Arg-12EG-S	4-Arg-12EG-S	8-Arg-12EG-S
Arg-2EG-Cl	2-Arg-2EG-Cl	4-Arg-2EG-Cl	8-Arg-2EG-Cl
Arg-3EG-Cl	2-Arg-3EG-Cl	4-Arg-3EG-Cl	8-Arg-3EG-Cl
Arg-4EG-Cl	2-Arg-4EG-Cl	4-Arg-4EG-Cl	8-Arg-4EG-Cl
Arg-6EG-Cl	2-Arg-6EG-Cl	4-Arg-6EG-Cl	8-Arg-6EG-Cl
Arg-12EG-Cl	2-Arg-12EG-Cl	4-Arg-12EG-Cl	8-Arg-12EG-Cl

4.C.5 Cell Culture

In this report, the following cells were used for tests: cell lines (Bovine aortic endothelial cells (BAEC),), primary cells (Rat smooth muscle cells (RSMC), Human umbilical vein endothelial cells (HUVEC) and stem cells (Rat Mesenchymal stem cells (MSC)). All the cells were grown exactly as the recommended ATCC protocols. For example, the RSMC was grown as recommended at 37 °C in 5 % CO₂ in Dulbecco's minimal essential medium (DMEM) supplemented with 10 % FBS and antibiotics. The cell lines were used from passages 6 to 12 and primary cells and stem cells were used from passages 2-5. Media was changed every 2 days. Cells were grown to 70 % confluence before splitting, harvesting or transfection.

4.C.6 Preparation of Plasmid DNA and Complexes of Arg-PEEA/DNA

The luciferase encoding reporter plasmids, COL (-772) /LUC and green fluorescence protein encoding reporter plasmid DNA (GFP) were all provided by Dr. Bo Liu's lab at Surgery Department of Wisconsin University at Madison. All plasmids were prepared using Qiagen endotoxin-free plasmid Maxi kits according to the supplier's protocol. The quantity and quality of the purified plasmid DNA was assessed by spectrophotometric analysis at 260 and 280 nm as well as by electrophoresis in 1% agarose gel. Purified plasmid DNA were resuspended in TAE (Tris-acetate-EDTA) buffer and frozen in -20 °C. The DNA solution obtained had a concentration around 1.5-2.0 mg/mL and was diluted to around 0.5 mg/mL before use.

The Arg-PEEA/DNA complexes were prepared by adding the plasmid DNA buffer solution into the freshly prepared Arg-PEEA PBS buffer solutions at a room

temperature to obtain a desirable Arg-PEEA to DNA weight ratio (WR). In this report, a wide range of WR (from 50 to 3,000) of Arg-PEEA to DNA was tested. The mixed solution was immediately and slightly vortex for several seconds and then equilibrated at an ambient condition for 20-30 minutes. All the Arg-PEEA solutions and Arg-PEEA/DNA complexes were freshly prepared and used within 4 hours.

4.C.7 Zeta Potential and Particle Size Measurements for Arg-PEEA/DNA Complexes

The charge property of the Arg-PEEA/DNA complexes was studied by zeta potential measurements. Arg-PEEA solutions (2 mg/mL) were prepared by dissolving Arg-PEEAs in 1X PBS buffer solution and the solution was filtered (0.45 μm pore size, Whatman®) before experiments. The Arg-PEEA/DNA complexes were prepared by adding the plasmid DNA (N3012, New England Lab) buffer solution of pre-determined amounts to the freshly made Arg-PEEA PBS buffer solutions (1mL volume total) to obtain the desirable Arg-PEEA to DNA weight ratio (WR). The mixed solution was immediately and slightly vortex for several seconds, and then equilibrated at an ambient condition for 20 minutes. After that, the zeta potential of the Arg-PEEA/DNA complexes was measured at 25 °C by using a Malvern Zetasizer Nano-ZS machine (Worcestershire, UK). Zeta potentials were calculated by using the Smoluchowsky model for aqueous suspensions. 2-Arg-6E-Cl was selected for this study and the zeta potentials of the 2-Arg-6E-Cl/DNA complexes at a series of WR were measured.

The particle sizes of the Arg-PEEA/DNA complex were studied by the same Malvern Instruments Zetasizer Nano ZS instrument, which used light scattering to

measure the average hydrodynamic radius of particles in solution. Samples were placed in 1.0 mL plastic cuvettes and three measurements consisting of 50 runs with 5 s duration were performed at 25 °C. The instrument was standardized with 1 mm polystyrene beads and particle size was reported as the average of the three measurements with an error measurement of one standard deviation. 2-Arg-6E-Cl was selected for this study and the particle sizes of the 2-Arg-6E-Cl/DNA complexes at a series of WR were measured.

4.C.8 Gene Transfection and Luciferase Assay

The complexes formed between plasmid DNA and the Arg-PEEAs were assessed for their *in vitro* transfection activity utilizing a transient expression of luciferase reporter in cells. First, the transfection protocol for Arg-PEEAs was studied and optimized in terms of cell type, cell density, buffer types, transfection time, transfection media, and temperature. After optimization, all transfection experiments were carried out according to the optimized protocol.

The details for the optimized transfection protocol for Arg-PEEAs are given below. For cell lines, such as rat aortic SMC A10 cells, the cells were seeded in 0.5 mL complete DMEM (10 % FBS, 1 % Hepes, 1 % penicillin-streptomycin) at 30×10^3 per well in a 24-well plate 24 hours before transfection (70 % confluent at transfection). Before transfection, the cell culture media was removed and the cells were washed with PBS buffer twice. Then 1.0 mL warmed serum free DMEM media (without antibiotics) was added into each well. For Lipofectamine2000[®], the media was used according to the manufacturer's recommendation. The formulated Arg-PEEA/DNA complex solution was then added into each well. The plasmid DNA

amount was fixed at 1 µg per well for 24-well cell culture plate. The transfection mixtures were immediately and slightly piped up and down for a few seconds, the cells were transfected for 4 h at 37 °C (5 % CO₂) in an incubator, and then the media solution was removed. After that, 0.5 mL of complete DMEM (10 % FBS, 1 % Hepes, 1 % penicillin-streptomycin) were added into each well and kept incubated at 37 °C (5 % CO₂) in an incubator. After 48 hours, cells were harvested for luciferase reading. Triplicate results were obtained in each data point. The main differences between transfection of cell lines and primary cells/stem cells were the transfection time and cell culture media. For transfection time: 4 h is needed for cell lines and 12-16 h is needed for primary and stem cells; for cell culture media, the transfection media is the media recommended by ATCC without serum, the medias before and after transfection are the medias recommended by ATCC.

Gene expression was determined by the luciferase activity using a DT 20/20 luminometer (Turner Biosystems, Sunnyvale, CA) with Dual Luciferase Assay System (Promega) according to the manufacturer's instruction. Luciferase assay was performed according to Promega's recommendation. Briefly, cells from each well of a 24-well plate were lysed in 100 µL lysis buffer, transferred to a micro-tube, and then centrifuged at 10,000 g for 2 min. Supernatants were collected and analyzed for luciferase activity. In a typical experiment, 20 µL of supernatant was added to luminometric tubes containing 100 µL of luciferase substrate (Promega). Light emission was measured with a Dual-luciferase detection system for periods of 5 sec, and the relative light units (RLUs) were determined. Triplicate results were used in each experiment. RLUs were normalized to the protein contents of each sample measured by spectrophotometric analysis.

4.C.9 Green Fluorescence Protein (GFP) Assay

To visually confirm the transfection obtained from the luciferase activity reading, we also transfected many types of cells with a plasmid DNA encodes for Green Fluorescent Protein (GFP). The transfection protocol was exactly the same as the one used for luciferase assay, except GFP encoded plasmid DNA was used. Following 48 h incubation after transfection, cells were examined under a fluorescence microscope (Nikon TE2000-U DIC inverted microscope with UV, GFP/FITC and Tx Red filter sets) for any GFP expression (cells showed green). The cell images were recorded from the random but typical fields of the cell culture wells.

4.C.10 Evaluation of Cytotoxicity of the Arg-PEEA/DNA Complexes

The evaluation of the cytotoxicity of the Arg-PEEA/DNA complexes was performed by MTT assay. All the cell types were tested for this study. The cultured cells were seeded at an appropriate cell density concentration (3,000 or 5,000 cells/well) in 96-well plates and incubated overnight in a 5 % CO₂ incubator at 37 °C. The cells were, then, treated with various Arg-PEEA/DNA complex solutions for 4 h or 12 h. The media was removed after that and complete DMEM was then added. The cells treated with normal cell culture media only were used as the negative control (NC). PEI and Lipofectamine2000[®] treated cells (same time as the Arg-PEEA/DNA complexes) were used as the positive controls. After 48 h incubation of the treated cells at 37 °C and 5 % CO₂, 15 µL of MTT solution (5 mg/mL) was added to each well, followed by 4 h incubation at 37 °C, 5 % CO₂. The cell culture medium including complex solution was carefully removed and 150 µL of acidic isopropyl alcohol (with 0.1 M HCl) was added to dissolve the formed formazan crystal. OD was

measured at 570 nm (subtract background reading at 690 nm) using a VersaMax Tunable Microplate reader. The cell viability (%) was calculated according to the following equation: Viability (%) = $(OD_{570 \text{ (sample)}} - OD_{620 \text{ (sample)}}) / (OD_{570 \text{ (control)}} - OD_{620 \text{ (control)}}) \times 100 \%$; where the $OD_{570 \text{ (control)}}$ represented the measurement from the wells treated with medium only, and the $OD_{570 \text{ (sample)}}$ from the wells treated with various Arg-PEEA and Arg-PEEA/plasmid DNA complexes. Thus, the cell viability was expressed as the percentage of the blank negative control. Triplicates were used in each experiment.

4.C.11 Statistics

Where appropriate, the data are presented as mean \pm standard error of the mean calculated over at least three data points. Significant differences compared to control groups were evaluated by unpaired Student's t-test or Dunnet test at p 0.05, and between more than two groups by Tukey's test with or without one-way ANOVA analysis of variance. JMP software (version 8.0, from SAS Company) was used for data analysis.

4.D Results and Discussions

The goal of this study was to examine a new generation of Arg-PEA, oligoethylene glycol based Arg-PEA (Arg-PEEA), for gene delivery applications, especially for the transfection of primary cells and stem cells, which are hard to be transfected with high efficiency and low cytotoxicity simultaneously. And we also want to examine the relationship of polymer structure-function so that we could have

more understanding for designing of advanced generations of non-viral gene delivery vectors.

In this paper, we focused on a few easily defined chemical structure parameters of Arg-PEEAs, i.e., x (the number of $-\text{CH}_2-$ groups in the diacid part) and y (the number of ethylene glycol, $-\text{CH}_2\text{CH}_2\text{O}-$ groups in the diol part). And how the introduction of ethylene glycol groups could affect Arg-PEEAs' properties and their transfection performance when compared with the 1st generation aliphatic diol based Arg-PEAs⁶. The data obtained in this study could advance the understanding of our prior reported preliminary biological evaluation and gene delivery of Arg-PEA based biomaterials⁶

The incorporation of oligoethylene glycols into Arg-PEAs is different from others' published studies of PEG involved gene transfection which have focused on the modification of the side chain of existing polymers (e.g, poly(L-lysine), chitosan and PEI) or making amphiphilic block copolymers(e.g, PLA, PLA-*b*-poly (L-lysine)) to increase the transfection efficiency or cell viability^{15, 29-31, 36, 37}. Based on our unpublished results, the very stiff backbone (with double bonds in the backbone instead of side chain) in the Arg-PEA main chain would cause significant decreasing of transfection efficiency of Arg-PEAs. Here we proposed that more flexible Arg-PEA backbone via oligoethylene glycol could have a better transfection performance.

4.D.1 Synthesis and Physicochemical Characterization of Arg-PEEAs

4.D.1.a Synthesis of monomers

In this study, the synthesis protocols of all the new monomers and Arg-PEEA polymers were optimized. Three types of di-*p*-nitrophenyl esters of dicarboxylic acids

(Monomer **I**, **NSu**, **NA** and **NS**) were synthesized here and the details of the synthesis and characterization of these Monomers **I** have been reported previously³⁹. The *p*-toluenesulfonic acid salts of L-arginine diester (**II**) from oligoethylene glycols, however, are newly developed for the first time. Twelve types of these new monomers **II** were prepared and the differences among these monomers **II** are the salt type (toluenesulfonic acid salt for the S type, and hydrochloride salt for the Cl type) and ethylene glycol unit length (*y*) in the diol part between the two adjacent ester groups: number of ethylene glycol units varies from 2 to 12. The chemical structures of these 12 types of Arg-based monomers **II** were all confirmed by ¹H-NMR, FTIR and solubility tests. All these new bis (L-arginine) diesters are very moisture sensitive and should be stored under vacuum at room temperature or below.

At room temperature, the solubility data showed that these bis (L-arginine) diesters have very good solubility in polar solvents, such as water, DMSO, DMF; but insoluble in non-polar or weak polar solvents, such as isopropanol, acetone, and ethyl acetate. However, it was found that isopropanol can dissolve monomers **II** at 50 °C or higher temperature (boiling point of isopropanol is 82.5 °C). The following are some ¹H-NMR and FTIR details for the Monomers **IIa** (S salt type). The ¹H-NMR data for the Monomers **IIb** (Cl salt type) is the same as the corresponding S salt type Monomer **IIa**, except for the difference of peak intensity of some groups:

Arg-2E-S: Yield of purified product: 81%. Appearance: amorphous white powder. IR (cm⁻¹): 1735 [-C(O)-], 1177 [-O-], 1127 [-CH₂-O-CH₂-]; ¹H-NMR (DMSO-d₆, ppm, δ): 1.61 [4H, -CH₂-CH₂-CH₂-NH-], 1.77 [4H, -OC(O)-CH(NH₃⁺)CH₂-(CH₂)₂-], 2.29 [6H, H₃C-Ph-SO₃⁻], 3.10 [4H, -(CH₂)₂-CH₂-NH-], 3.60 [4H, -(O)C-O-CH₂-CH₂-O-],

4.07 [2H, $^+\text{H}_3\text{N}-\text{CH}(\text{R})-\text{C}(\text{O})-\text{O}-$], 4.32 [4H, $-(\text{O})\text{C}-\text{O}-\text{CH}_2-$], 7.13, 7.48 [16H, Ph], 7.59 [10H, $-\text{CH}_2-\text{NH}(\text{NH}_2^+)-\text{NH}_2$], 8.42 [6H, $^+\text{H}_3\text{N}-\text{CH}(\text{R})-\text{C}(\text{O})-\text{O}-$];

Arg-3E-S: Yield of purified product: 85%. Appearance: amorphous white powder. IR (cm^{-1}): 1736 [$-\text{C}(\text{O})-$], 1178 [$-\text{O}-$], 1125 [$-\text{CH}_2-\text{O}-\text{CH}_2-$]; ^1H NMR (DMSO- d_6 , ppm, δ): 1.63 [4H, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-$], 1.78 [4H, $-\text{OC}(\text{O})-\text{CH}(\text{NH}_3^+)\text{CH}_2-(\text{CH}_2)_2-$], 2.28 [6H, $\text{H}_3\text{C}-\text{Ph}-\text{SO}_3-$], 3.12 [4H, $-(\text{CH}_2)_2-\text{CH}_2-\text{NH}-$], 3.55-65 [8H, $-(\text{O})\text{C}-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-$], 4.09 [2H, $^+\text{H}_3\text{N}-\text{CH}(\text{R})-\text{C}(\text{O})-\text{O}-$], 4.31 [4H, $-(\text{O})\text{C}-\text{O}-\text{CH}_2-$], 7.15, 7.49 [16H, Ph], 7.62 [10H, $-\text{CH}_2-\text{NH}(\text{NH}_2^+)-\text{NH}_2$], 8.47 [6H, $^+\text{H}_3\text{N}-\text{CH}(\text{R})-\text{C}(\text{O})-\text{O}-$];

Arg-4E-S: Yield of purified product: 87%. Appearance: amorphous white powder. IR (cm^{-1}): 1734 [$-\text{C}(\text{O})-$], 1179 [$-\text{O}-$], 1124 [$-\text{CH}_2-\text{O}-\text{CH}_2-$]; ^1H NMR (DMSO- d_6 , ppm, δ): 1.62 [4H, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-$], 1.79 [4H, $-\text{OC}(\text{O})-\text{CH}(\text{NH}_3^+)\text{CH}_2-(\text{CH}_2)_2-$], 2.27 [6H, $\text{H}_3\text{C}-\text{Ph}-\text{SO}_3-$], 3.11 [4H, $-(\text{CH}_2)_2-\text{CH}_2-\text{NH}-$], 3.60-70 [12H, $-(\text{O})\text{C}-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-$], 4.08 [2H, $^+\text{H}_3\text{N}-\text{CH}(\text{R})-\text{C}(\text{O})-\text{O}-$], 4.30 [4H, $-(\text{O})\text{C}-\text{O}-\text{CH}_2-$], 7.17, 7.50 [16H, Ph], 7.63 [10H, $-\text{CH}_2-\text{NH}(\text{NH}_2^+)-\text{NH}_2$], 8.49 [6H, $^+\text{H}_3\text{N}-\text{CH}(\text{R})-\text{C}(\text{O})-\text{O}-$];

Arg-6E-S: Yield of purified product: 89 %. Appearance: amorphous white viscous solid. IR (cm^{-1}): 1737 [$-\text{C}(\text{O})-$], 1177 [$-\text{O}-$], 1127 [$-\text{CH}_2-\text{O}-\text{CH}_2-$]; ^1H NMR (DMSO- d_6 , ppm, δ): 1.63 [4H, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-$], 1.80 [4H, $-\text{OC}(\text{O})-\text{CH}(\text{NH}_3^+)\text{CH}_2-(\text{CH}_2)_2-$], 2.29 [6H, $\text{H}_3\text{C}-\text{Ph}-\text{SO}_3-$], 3.14 [4H, $-(\text{CH}_2)_2-\text{CH}_2-\text{NH}-$], 3.60-70 [20H, $-(\text{O})\text{C}-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-(\text{CH}_2-\text{CH}_2)_2$], 4.10 [2H, $^+\text{H}_3\text{N}-\text{CH}(\text{R})-\text{C}(\text{O})-\text{O}-$], 4.32 [4H, $-(\text{O})\text{C}-\text{O}-\text{CH}_2-$], 7.16, 7.50 [16H, Ph], 7.64 [10H, $-\text{CH}_2-\text{NH}(\text{NH}_2^+)-\text{NH}_2$], 8.50 [6H, $^+\text{H}_3\text{N}-\text{CH}(\text{R})-\text{C}(\text{O})-\text{O}-$];

Arg-12E-S: Yield of purified product: 84 %. Appearance: amorphous white viscous solid. IR (cm^{-1}): 1737 [$-\text{C}(\text{O})-$], 1177 [$-\text{O}-$], 1124 [$-\text{CH}_2-\text{O}-\text{CH}_2-$]; ^1H NMR (DMSO-

d6, ppm, δ): 1.61 [4H, -CH₂-CH₂-CH₂-NH-], 1.78 [4H, -OC(O)-CH(NH₃⁺)CH₂-(CH₂)₂-], 2.29 [6H, H₃C-Ph-SO₃-], 3.10 [4H, -(CH₂)₂-CH₂-NH-], 3.60-70 [44H, -(O)C-O-CH₂-CH₂-O-(CH₂-CH₂)₅], 4.09 [2H, ⁺H₃N-CH(R)-C(O)-O-], 4.31 [4H, -(O)C-O-CH₂-], 7.15, 7.49 [16H, Ph], 7.61 [10H, -CH₂-NH(NH₂⁺)-NH₂], 8.47 [6H, ⁺H₃N-CH(R)-C(O)-O-];

4.D.1.b Synthesis of Arg-PEEA Polymers

One major distinction of the Arg-PEEAs (Figure 4.2) synthesized in this study is that they are in the *p*-toluenesulfonic acid salt or chlorine salt form, while all other PEAs from prior reported studies were not in any salt form^{38, 40-46}. The formation of salt in these Arg-PEEAs is because of the strong base nature of the guanidine group in L-Arginine. The guanidine group has a much higher 12.5 pKa value than the amine groups of PEI and PLL-HBr, suggesting a stronger interaction with anionic DNA chain. The *p*-toluenesulfonic acid or hydrochloride acid counter ions, however, were found not to adversely affect the DNA binding capability of Arg-PEEAs, and the following cytotoxicity tests showed that all the Arg-PEEAs are nontoxic to the cells even at large dosages.

The reaction conditions for the Arg-PEEA synthesis were optimized in terms of reaction temperature and time, catalyst and its concentration, the molar ratio between 2 monomers, and monomer concentrations. The optimal polycondensation reaction conditions for the Arg-PEEAs are: reaction temperature: 75 °C; duration: 48 h, concentration of each monomer: 1.0-1.5 mol/L; DMSO solvent; catalyst (acid acceptor): NEt₃. The molar ratio of the two monomers (**I** and **IIa or b**) should be exactly equal to 1: 1, and the molar ratio between the monomer and acid receptor must

be 1.0: 1.1. The final product yields are high (> 80%) under the optimized reaction conditions.

For the chemical structure identification of all the synthesized Arg-PEEAs, their structures were confirmed by both ^1H -NMR and FTIR spectra. For FTIR data, the carbonyl bands at $1648\text{--}1650\text{ cm}^{-1}$ (amide I), $1538\text{--}1542\text{ cm}^{-1}$ (amide II), and $1738\text{--}1742\text{ cm}^{-1}$ (ester), and NH vibrations at 3290 cm^{-1} are typical for all Arg-PEEAs obtained. Figure 4.6 showed an example of the ^1H -NMR spectrum of 2-Arg-2E-S. All the ^1H -NMR peaks of 2-Arg-2E-S were well identified, and the integration area ratio is consistent with the calculated theoretical ratio. The ^1H -NMR peaks marked with numbers from 1 to 12 are assigned to the corresponding protons of 2-Arg-2E-S as shown in Figure 4.6.

For the thermal property of the Arg-PEEAs, DSC results indicated that the Arg-PEEAs did not have melting points (T_m). For the glass transition temperature (T_g) of Arg-PEEAs, an examination for the effect of the number of methylene groups in the diacid part (x) of the Arg-PEEAs revealed that an increase in x at a constant y led to a decrease in T_g . For example, at $y = 2$, the T_g decreased from $31\text{ }^\circ\text{C}$ (2-Arg-2E-S) to $29\text{ }^\circ\text{C}$ (4-Arg-2E-S) then to $25\text{ }^\circ\text{C}$ (8-Arg-2E-S), when x decreased from 2, 4 to 8. When the y value was increased from 2 to 12 at a fixed x value, the same decreasing trend in T_g was observed.

According to our unpublished data, unsaturated Arg-PEAs (double bonds in the PEA backbone) would significantly increase the T_g value because of the stiff polymer backbone. For example, 2-Arg-2-S has a T_g around $52\text{ }^\circ\text{C}$, while 2-UArg-2-S has a T_g around $112\text{ }^\circ\text{C}$. Therefore, the introduction of more flexible segments like

oligoethylene glycol in this study to the PEA polymer backbone would be expected to make the whole Arg-PEEA polymer chain structure much more flexible and lower T_g than those Arg-PEA having fatty diols. For example, the T_g of Arg-PEEA (2-Arg-2E-S) is near 50% lower than the Arg-PEA (2-Arg-4-S), 31 °C vs. 46 °C, and the only chemical structure difference between 2-Arg-2E-S and 2-Arg-4-S is an extra oxygen atom from the ethylene glycol per each repeating unit in the 2-Arg-2E-S. Thus, the introduction of a flexible oligoethylene glycol moiety significantly decreased the T_g value in Arg-PEEAs.

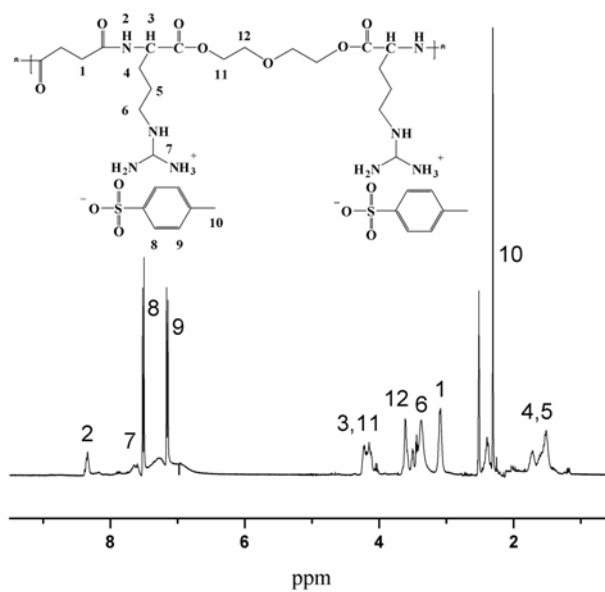


Figure 4.6 ¹H-NMR spectra of 2-Arg-2E-S

The solubility of Arg-PEEAs in water and common organic solvents at room temperature was tested. Solubility was assessed at 2.0 mg/mL at a room temperature as an index whether a polymer is soluble or not. Due to their strong polar nature, Arg-PEEAs tended to dissolve in polar solvents (Table 4.3). All of the Arg-PEEAs synthesized were soluble in polar organic solvents like DMSO, DMF, methanol or water, but did not dissolve in non-polar or weak polar organic solvents like ethyl acetate, THF or chloroform. The effect of x and y material parameters on Arg-PEEA water solubility revealed that both x and y had a major impact on the water solubility of Arg-PEEAs; and an increase in the methylene chain length in the dicarboxylic acid part (x) reduced the water solubility significantly due to the increasing hydrophobicity. For example, the solubility of Arg-PEEAs decreased from 200 mg/mL to 15 mg/mL as x increased from 2 (2-Arg-2E-S) to 8 (8-Arg-2E-S) at a constant y=2.

A similar solubility – structure relationship was also found with an increase in y (from 2 to 6 in the diol segment) at a constant x. However, the relationship broke down at a large y value, such as y =12. Thus, the water solubility could be used as an index of hydrophilicity vs. hydrophobicity of Arg-PEEA polymers. By adjusting the x or y, the hydrophilicity of Arg-PEEA polymers can be shifted toward more hydrophilic or hydrophobic for meeting specific needs.

Compared with the saturated aliphatic diol-based Arg-PEAs, Arg-PEEAs showed a significant increase in water solubility due to the introduction of relative hydrophilic and flexible ethylene glycol units. For example, the 2-Arg-2E-S showed much higher water solubility than 2-Arg-4-S, and the solubility difference is more than 150 mg/mL as the only chemical structure difference of these two polymers is one oxygen atom in the repeating unit. All the prepared Arg-PEEAs were obtained in fairly good yields (>

75 %) with η_{red} (reduced viscosity) ranging from 0.11 to 0.39 dL/g (Table 4.3). The molecular weight data of Arg-PEEA were not available because all arginine based PEAs cannot be dissolved in THF, which is the only solvent for the central GPC facility available to us.

4.D.2 Gel Retardation Assay

Gel Retardation Assay is a widely used method for measuring DNA condensing capability of polymeric transfection candidates. The DNA condensation capability of a transfection agent is known to have a profound effect on the subsequent gene delivery efficiency, but is not the only factor that is responsible for the outcome of gene delivery efficiency. In this study, the main goal is to determine the proper weight ratio (WR) of Arg-PEEA to DNA required for a completely condensing of DNA during the polyplex formation, the first key step toward non-viral gene transfection.

Figures 4.7 and 4.8 showed some examples of the electrophoresis data for the Arg-PEEA/DNA complexes. These results demonstrated the DNA condensation capability of Arg-PEEAs, and provided the basic formulation information for subsequent transfection experiments. Most important of all, the electrophoresis data showed that different types of Arg-PEEAs (in terms of x and y parameters) required different amounts of Arg-PEEAs for a complete DNA condensation as indicated by the different WR.

Table 4.3 Water solubility and reduced viscosity (in DMSO) at room temperature of Arg-PEEAs (x-Arg-yEG-z), part A

Arg-PEEA	S(mg/mL)	η_{red} (dL/g)
2-Arg-2EG-S	200±10	0.15±0.01
2-Arg-3EG-S	100±10	0.19±0.03
2-Arg-4EG-S	40±5	0.13±0.01
2-Arg-6EG-S	40±5	0.21±0.01
2-Arg-12EG-S	100±10	0.26±0.02
4-Arg-2EG-S	100±10	0.25±0.01
4-Arg-3EG-S	60±5	0.21±0.02
4-Arg-4EG-S	15±2	0.29±0.01
4-Arg-6EG-S	15±2	0.27±0.01
4-Arg-12EG-S	20±2	0.35±0.03
8-Arg-2EG-S	15±2	0.27±0.03
8-Arg-3EG-S	10±2	0.25±0.01
8-Arg-4EG-S	6±1	0.35±0.02
8-Arg-6EG-S	6±1	0.32±0.01
8-Arg-12EG-S	10±2	0.36±0.02

Table 4.3 Water solubility and reduced viscosity (in DMSO) at room temperature of Arg-PEEAs (x-Arg-yEG-z), part B

Arg-PEEA	S(mg/mL)	η_{red} (dL/g)
2-Arg-2EG-Cl	200±10	0.13±0.02
2-Arg-3EG-Cl	200±10	0.11±0.01
2-Arg-4EG-Cl	80±5	0.17±0.02
2-Arg-6EG-Cl	60±5	0.19±0.01
2-Arg-12EG-Cl	100±10	0.20±0.01
4-Arg-2EG-Cl	150±10	0.21±0.02
4-Arg-3EG-Cl	100±10	0.25±0.03
4-Arg-4EG-Cl	50±5	0.20±0.01
4-Arg-6EG-Cl	30±2	0.22±0.02
4-Arg-12EG-Cl	50±5	0.19±0.01
8-Arg-2EG-Cl	40±5	0.35±0.01
8-Arg-3EG-Cl	30±5	0.30±0.01
8-Arg-4EG-Cl	15±2	0.23±0.01
8-Arg-6EG-Cl	10±2	0.33±0.01
8-Arg-12EG-Cl	15±2	0.39±0.01

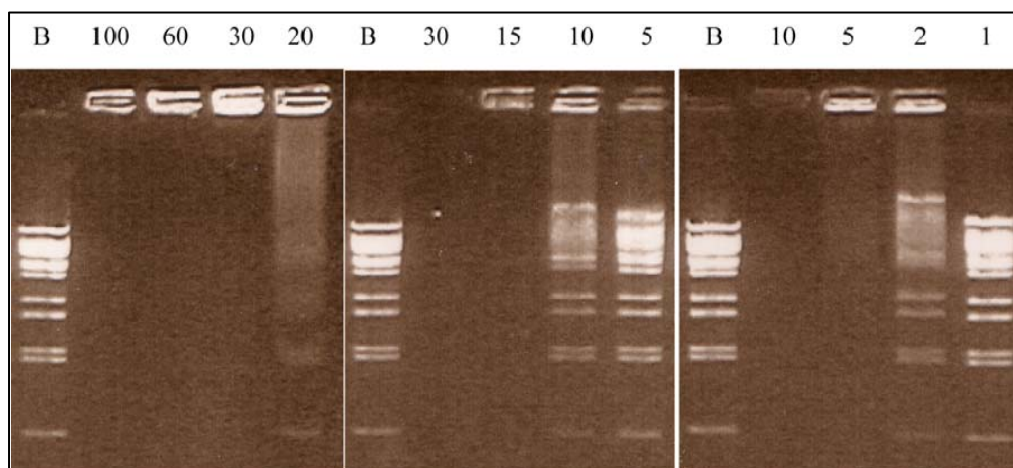


Figure 4.7 Effect of methylene chain length (x) of Arg-PEEAs on their condensation ability to DNA: B means blank, (only 1 μ g N3014S DNA, no Arg-PEEA); the other numeric numbers are the weight ratio of Arg-PEEA to DNA. The Arg-PEAs tested are: 2-Arg-2E-Cl, 4-Arg-2E-Cl, 8-Arg-2E-Cl (from left to right)

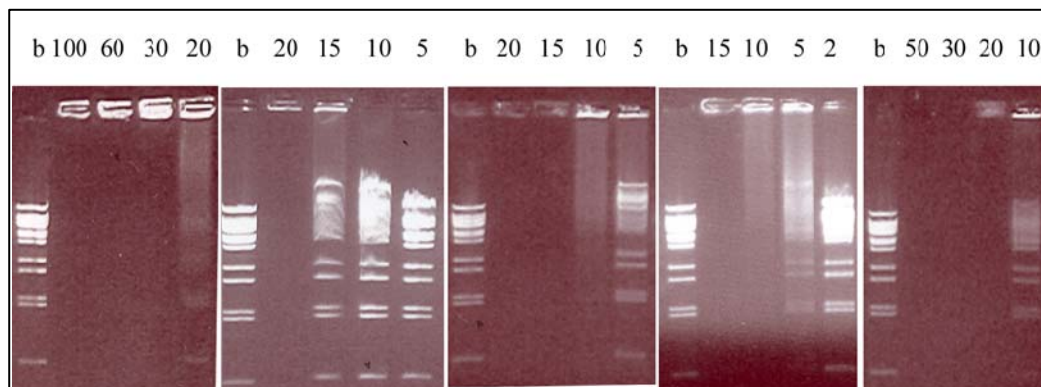


Figure 4.8 Effect of oligoethylene glycol chain length (y) of Arg-PEEAs on the condensation ability to DNA: B means blank, (only 1 μ g N3014S DNA, no Arg-PEEA); the other numbers are the WR of Arg-PEEA to DNA. The Arg-PEAs are: 2-Arg-2E-Cl, 2-Arg-3E-Cl, 2-Arg-4E-Cl, 2-Arg-6E-Cl and 2-Arg-12E-Cl (from left to right)

In order to have a better quantitative comparison of the DNA condensation capability of Arg-PEEAs, the minimum WR of Arg-PEEA to DNA that could completely condense DNA was selected and compared. For example (Figure 4.7), 2-Arg-2E-Cl needed a minimum WR of 30 to completely condense the DNA marker; while 4-Arg-2E-Cl and 8-Arg-2E-Cl needed a minimum WR of 15 and 10 for a complete condensation, respectively. Thus, the minimum WR required for a complete DNA condensation decreased when the x value in the Arg-PEEA increased. The same trend was also observed when y value was increased from 2 to 4. This relationship, however, did not hold at a large y values, such as y=6 and 12 (Figure 4.8).

We also found that the Arg-PEEA buffer solutions, if stored at 4 °C, could retain their DNA condensing capability for about 1 month or even longer time, suggesting there was no obvious structure change or degradation of Arg-PEEAs in the buffer solution at 4 °C. It is important to recognize that precise Arg-PEEA polymer concentration and volume, and a complete Arg-PEEAs dissolution are critical for reproducible transfection experiments. In this study, the Arg-PEEA polymers must be dissolved completely and the volume should be in the range of 2-5 μ L to avoid any possible experimental errors. Some Arg-PEEAs have lower water solubility and would take a long time for a complete dissolution.

4.D.3 Transfection Efficiency

In this paper, the plasmid DNA that encodes for a firefly luciferase driven by a collagen promoter was used. By measuring luciferase activities in cell lysates, which in this case is mainly determined by the amounts of DNA transferred into the cells, we compared the transfection efficiency of Arg-PEEAs with a commercial transfection

agent, Lipofectamine2000[®], for determining the transfection feasibility of Arg-PEEAs.

In any transfection protocol development, cell density, transfection time, transfection temperature, transfection media and buffer types are important parameters for optimization to achieve the best transfection data. In the Arg-PEEA/DNA system, the optimized transfection protocol of Arg-PEEA/DNA system was: transfection time: 3-4 h for cell lines and 12-16 h for primary cells and stem cells; transfection temperature: 37 °C; transfection media: serum free DMEM media without antibiotics; buffer for Arg-PEEA/DNA: HEPES (20 mM) or PBS buffer (1X); cell density: 10,000-30,000 per well for 24-well cell culture plate. At this optimized condition, it was observed that the luciferase activity could reach the peak value over a range of WR of Arg-PEEA to DNA.

Figure 4.9 showed an example of the transfection results from 4 types of Arg-PEEA/DNA at various WR: 2-Arg-4E-S, 2-Arg-6E-S, 2-Arg-4E-Cl and 2-Arg-6E-Cl. The data show that all the Arg-PEEA/DNA could show good transfection ability over a very broad WR range, and each type of Arg-PEEA/DNA showed a peak transfection at a specific WR as indicated by a maximum bell curve on one of the 4 Arg-PEEAs as an example. The 2-Arg-6E-Cl/DNA showed transfection capability over WR from 200 to 2000, but the highest transfection capability was around the WR of 1000. For the 2-Arg-4E-S/DNA system, the peak transfection, however, occurred at WR 500.

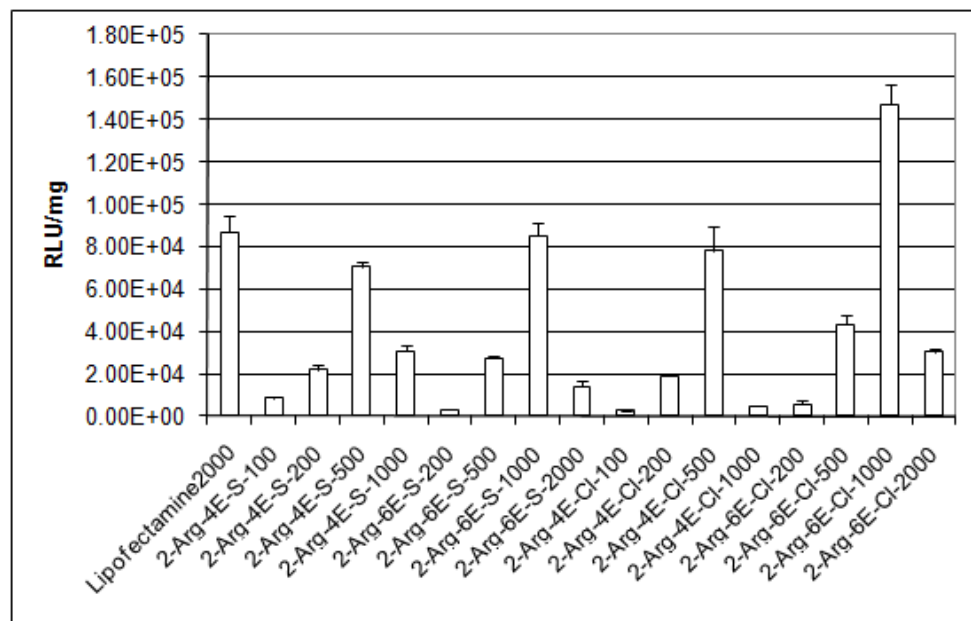


Figure 4.9 Effects of type of Arg-PEEAs and weight ratio of Arg-PEEAs to DNA on transfection efficiency of Arg-PEEA/DNA complexes expressed by firefly luciferase activity. The cells used here were primary rat smooth muscle cells (RSMC). Plasmid DNA used were COL (-772)/Luc. Lipofectamine2000[®] was tested with the suggested optimum WR to DNA by Invitrogen. The numeric numbers at the end of each polymer sample label are the weight ratio of polymer to DNA.

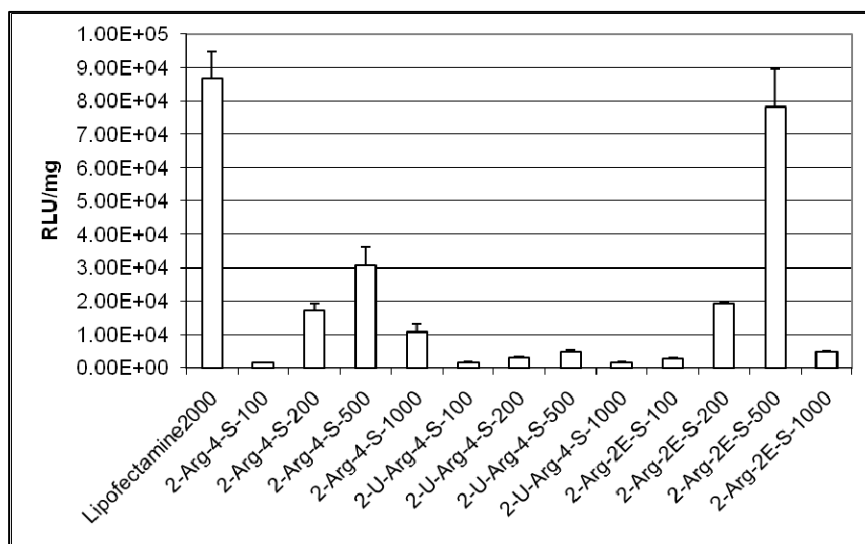


Figure 4.10 Effects of rigidity/flexibility of Arg-PEA backbone and weight ratio of Arg-PEAs to DNA on transfection efficiency of Arg-PEA/DNA complexes expressed by firefly luciferase activity. The cells used here were primary rat smooth muscle cells (RSMC). Plasmid DNA used were COL (-772)/Luc. The numeric numbers at the end of each polymer sample label are the weight ratio of polymer to DNA.

Table 4.4 Relative transfection efficiency of Arg-PEEAs

2-Arg-2EG-S 105	2-Arg-3EG-S 112	2-Arg-4EG-S 85	2-Arg-6EG-S 97	2-Arg-12EG-S 78
4-Arg-2EG-S 21	4-Arg-3EG-S 35	4-Arg-4EG-S 44	4-Arg-6EG-S 41	4-Arg-12EG-S 17
8-Arg-2EG-S 73	8-Arg-3EG-S 115	8-Arg-4EG-S 107	8-Arg-6EG-S 81	8-Arg-12EG-S 69
2-Arg-2EG-Cl 87	2-Arg-3EG-Cl 130	2-Arg-4EG-Cl 91	2-Arg-6EG-Cl 195	2-Arg-12EG-Cl 75
4-Arg-2EG-Cl 31	4-Arg-3EG-Cl 27	4-Arg-4EG-Cl 14	4-Arg-6EG-Cl 39	4-Arg-12EG-Cl 22
8-Arg-2EG-Cl 103	8-Arg-3EG-Cl 98	8-Arg-4EG-Cl 94	8-Arg-6EG-Cl 140	8-Arg-12EG-Cl 75

These transfection results also showed that the WR of Arg-PEEAs to DNA reaching the optimum transfection efficiency was much higher than the minimal WR required for completely condensing DNA in the electrophoresis data. For example, 2-Arg-6E-Cl required a WR of 20 for a completely condensing DNA, but needed a WR of 1000 for a maximum DNA transfection. This finding may be attributed to the need of excess amounts of Arg-PEEAs to achieve not only a stable Arg-PEEA/DNA complex system in the transfection media but also provided additional cationic charge to the Arg-PEEA/DNA complex for its proper penetration into the cells membranes. The larger dosages of Arg-PEEA required for the optimized transfection, however, didn't impose any adverse cytotoxicity as described later.

To compare the transfection efficiency of all the Arg-PEEAs, the highest or peak RLU/mg (relative light unit/mg) of each polymer was selected and normalized against the RLU/mg value of the commercial control (Lipofectamine2000[®]), i.e., setting the RLU/mg value of the control at 100 (Table 4.4). This normalization process removed the batch to batch variation. The normalized transfection data in Table 4.4 showed that many of these Arg-PEEAs had comparable or better transfection efficiency (i.e., those Arg-PEEAs having 100 or greater normalized values) than the commercial transfection reagent Lipofectamine2000[®]. Those Arg-PEEAs having the low or high x (2, 3 and 8) and low and medium y (2, 3 and 6) values in this study showed one of the most favorable high transfection efficiency. For those Arg-PEEAs having medium x (4)value, regardless of y value and type of salts, would provided the poorest transfection. The reason is not very clear and further study may focus on the investigation of polymer structure. Those Arg-PEEAs having, the highest y value, (y=12), also showed the poorest transfection relative to Lipofectamine2000[®]. The

reason could be due to the significantly reduced charge density and further investigation is need.

In the introduction, we hypothesized that more flexible Arg-PEA backbone would be, a better transfection would be achieved. As a result, an introduction of a stiff Arg-PEA backbone should lead to a reduction in transfection efficiency of Arg-PEAs. In order to prove this hypothesis, we engineered unsaturated Arg-PEAs (UArg-PEA) to provide rigid backbone and examined their transfection level in a luciferase assay. In this report, we chose the 2-UArg-4-S, which had unsaturated $>C=C<$ double bonds in the diacid segment of the UArg-PEA backbone, as the most rigid Arg-PEA and examined its transfection efficiency against two other more flexible Arg-based PEAs: 2-Arg-4-S and 2-Arg-2E-S. These three types of Arg-based PEAs have very similar repeating unit formula (e.g., same x and y) and molecular weight, charge density and molecular weight. The repeating unit formula difference between 2-UArg-4-S and 2-Arg-4-S is 2 hydrogen atoms, while the repeating unit formula difference between 2-Arg-2E-S and 2-Arg-4-S is one oxygen atom. Therefore, the only main difference among these three Arg-based PEAs is the rigidity of their polymer backbone, which has also been verified by their T_g data in the previous thermal property discussion part. The T_g of 2-Arg-2E-S, 2-Arg-4-S and 2-UArg-4-S are 31 °C, 46 °C and 94 °C, respectively. We could find that the T_g difference between 2-Arg-4-S and 2-UArg-4-S is 48 °C, which is much bigger than the T_g difference between 2-Arg-4-S and 2-Arg-2E-S (15 °C difference)

Figure 4.10 showed the comparison of transfection efficiency among these three PEAs: 2-Arg-2E-S, 2-Arg-4-S and 2-UArg-4-S. The data in Figure 4.10 show that all of these three Arg-based PEAs reached a peak transfection efficiency value at the

weight ratio of Arg-PEA/DNA of 500. However, the 2-UArg-4-S showed lowest peak transfection value (i.e., the poorest transfection efficiency); while the 2-Arg-2E-S showed the highest value (best transfection efficiency). These differences in peak transfection data are consistent with the T_g data of these 3 Arg-based PEAs. The 2-Arg-2E-S has the lowest T_g (most flexible backbone chain), while 2-UArg-4-S has the highest T_g (most rigid backbone chain). Therefore, we could conclude that Arg-PEA with the most flexible chain could show the best transfection efficiency, while the least flexible chain could show the worst transfection efficiency. All the above data and discussion strongly supported our hypothesis about the relationship between Arg-PEA chain structure and transfection efficiency.

4.D.4 GFP Expression

To visually confirm the transfection efficiency obtained from the luciferase activity data, all the cells [Bovine aortic endothelial cell lines (BAEC), primary cells (Rat smooth muscle cells (RSMC) and Human umbilical vein endothelial cells (HUVEC)) and stem cells (Rat Mesenchymal stem cells (MSC))] were transfected by plasmid DNAs encoding for green fluorescent protein (GFP). Two days following the transfection, the cells were examined under a fluorescence microscope for their GFP expression (transfected cells would show green). Figure 4.10 shows that the GFP plasmid DNAs were successfully expressed inside different cell types (right pane) as commercial transfection agent Lipofectamine2000[®] did (left pane). And the BAEC cells treated by Lipofectamine2000[®] showed some morphology change, while the Arg-PEA treated cells did not show any obvious changes.

4.D.5 Zeta Potential and Particle Size Measurements for Arg-PEEA/DNA Complex

The Zeta potential measurement was used to study the charge property and the charge-structure relationship of the Arg-PEEA/DNA complex. Figure 4.11 showed the zeta potentials of some Arg-PEEA/DNA complexes as a function of the weight ratio of Arg-PEEA to DNA. The data in Figure 4.11 (2-Arg-6E-Cl) could be divided into 3 regions, depending on the weight ratio of Arg-PEEA to DNA. As the weight ratio of Arg-PEEA to DNA increased, the zeta potential of the complex increased (from negative to positive), suggesting that as more Arg-PEEAs added into the DNA, the charge property of the complex changed from negative to positive. A further increase in the weight ratio of Arg-PEEA to DNA, the zeta potential of the complex reached a peak, (WR is around 1000), and a further increase in the WR resulted in a reduction in zeta potential of the complex. The WR with a peak zeta potential suggests that the Arg-PEEA/DNA complex must be in the most stable state, and should be the optimal condition for gene transfection, which is consistent with transfection data, especially for the optimized WR data (Figures 7 and 8).

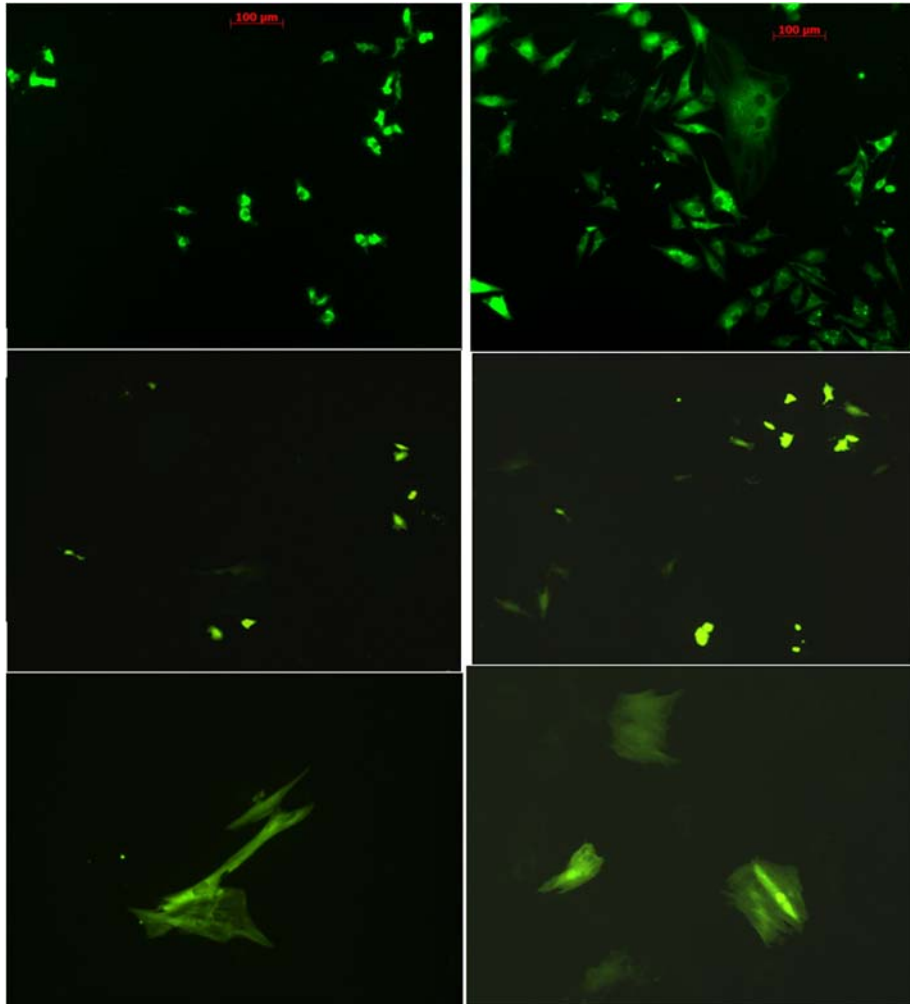


Figure 4.11 GFP expression of transfected cells under fluorescence microscope (10X). Green cells are the cells successfully transfected with GFP DNA. The cells transfected by lipofectamine2000[®] were used as controls (left) and by Arg-PEEA (2-Arg-6E-Cl, WR=1,000) (right). The cell types from top to bottom are: bovine aortic endothelial cells (BAEC), rat primary smooth muscle cells (RSMC) and rat mesenchymal stem cells (MSC); 4 h treatment for cell lines and 12 h treatment for primary and stem cells, and images were taken 48 h after transfection treatment.

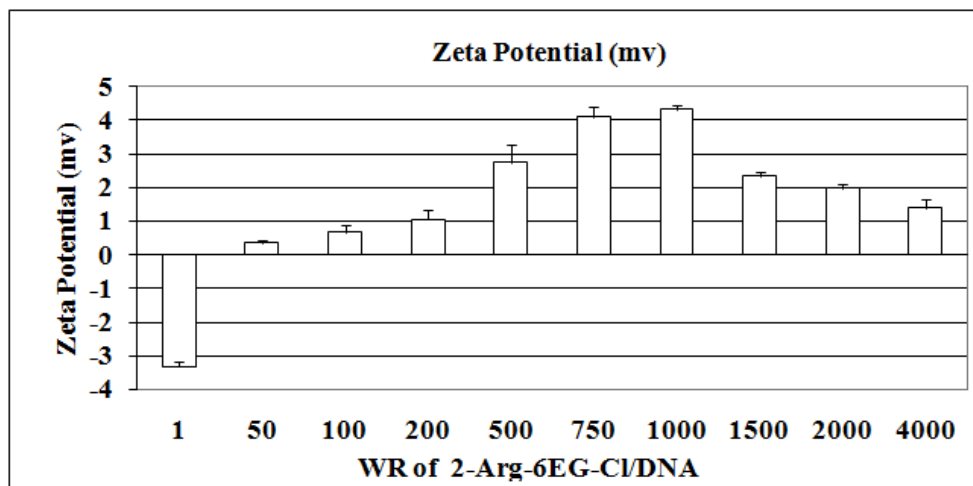


Figure 4.12 Zeta potential of 2-Arg-6E-Cl/DNA complex over a very wide weight ratio (WR) range. Positive value means the complex is positively charged; while negative value means the complex is negatively charged.

The particle size measurement was used to study the particle size of the Arg-PEEA/DNA complex in the buffer solution and the size-structure relationship of the Arg-PEEA/DNA complex. Figure 4.12 showed the particle sizes of some Arg-PEEA/DNA complexes from 2-Arg-6E-Cl as a function of the weight ratio of 2-Arg-6E-Cl to DNA. The particle size data in Figure 4.12 could be divided into 3 regions, depending on the ratio of Arg-PEEA to DNA. As the weight ratio of Arg-PEEA to DNA increased, the particle size of the complex decreased, suggesting that as more Arg-PEEAs added into the DNA, the DNA molecules were going to collapse. A further increase in the weight ratio of Arg-PEEA to DNA, the particle size of the complex reached a minimum value, (WR is around 1000), and a further increase in the WR resulted in an increase in particle size of the complex. For the relationship between Figures 4.11 and 4.12, the data from the 2 figures were very consistent, when the complex had the larger particle size, the zeta potential value was smaller, both of

them mean that the complex was in the more unstable state. The highest zeta potential value is corresponding to the smallest particle size and the complex is in the most stable state under this weight ratio. The WR for achieving a smallest particle size (Figure 4.12) suggests that the Arg-PEEA/DNA complex must be in the most stable state, and should be the optimal condition for gene transfection, which is also consistent with transfection data, especially for the optimized WR data.

4.D.6 Cytotoxicity of Arg-PEEA/DNA Complex by MTT Assay

Cytotoxicity of Arg-PEEA/DNA complexes was evaluated by MTT assay. The MTT system is a simple, accurate, reproducible means of detecting living cells via mitochondrial dehydrogenase activity. An increase in cell number (cell proliferation) results in an increase in the amount of MTT formazan production and hence an increase in UV absorbance. PEI, Lipofectamine2000[®] were used as the controls. All the synthesized Arg-PEEAs at different WR of Arg-PEEA/DNA were tested by MTT assay and some of the results were shown in Figure 4.13. Three types of cells were used for MTT assay and they were BAEC, RSMC Primary and MSC. The MTT data clearly demonstrated that at 12 h treatment, all the Arg-PEEA/DNA complexes showed very little toxicity to the tested cells even at a very large dosage. The statistical data analysis showed that there is no significant difference of any Arg-PEEA treatment compared to the control at the p value of 0.05 level by Dunnet test of planned comparison. So there is no evidence of toxicity of Arg-PEEAs.

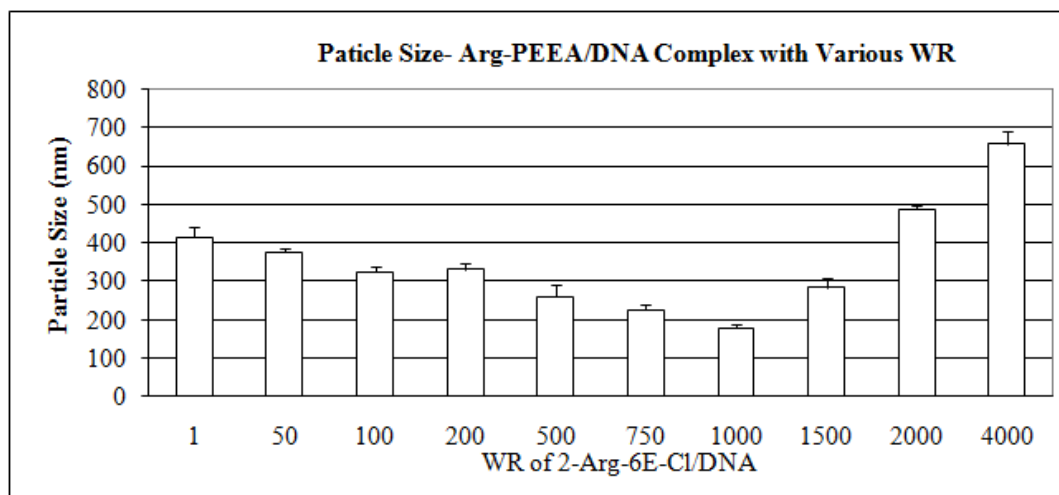


Figure 4.13 Particle size measurements of 2-Arg-6E-Cl/DNA complex over a very wide weight ratio (WR) of 2-Arg-6E-Cl to DNA

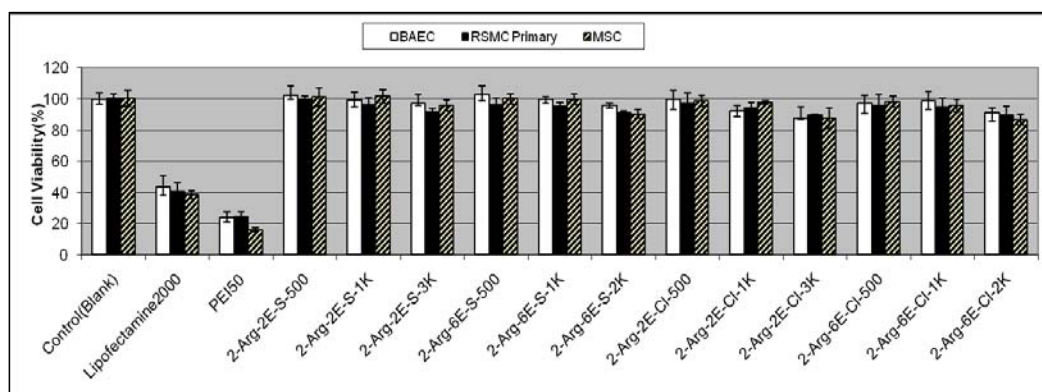


Figure 4.14 MTT cytotoxicity of 4 types of Arg-PEEA (2-Arg-2E-S, 2-Arg-6E-S, 2-Arg-2E-Cl, 2-Arg-6E-Cl) and their DNA complexes over a wide range of weight ratio of Arg-PEEA to DNA. Negative control (NC) is cells only without any transfection agent treatment. Lipofectamine2000[®] and PEI were used as the positive controls. The numbers after the PEI and Arg-PEEAs indicate the corresponding weight ratio of polymer to DNA. BAEC, RSMC primary cells and Rat Mesenchymal stem cells (MSC) cells were tested.

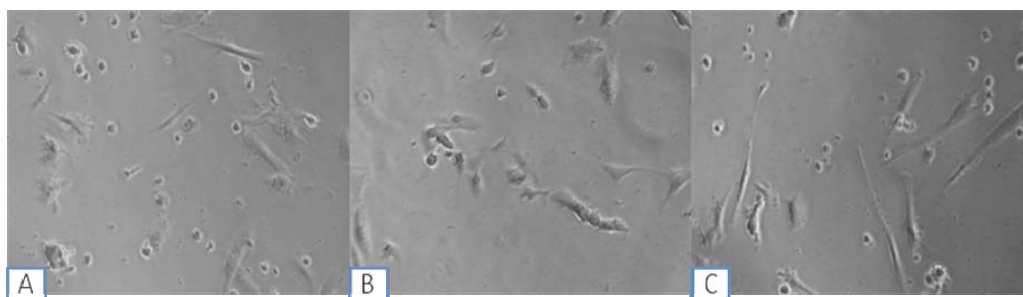


Figure 4.15 Human umbilical vein endothelial cells (HUVEC) morphology (10 x, 12 h treatment, after 48 h): (A) Negative control HUVEC, no polymer added; (B) HUVEC cells with 2 μ L Liopfectamine2000[®] and 1 μ g DNA added; (C) HUVEC cells with 1,000 μ g 2-Arg-6E-Cl and 1 μ g DNA added.

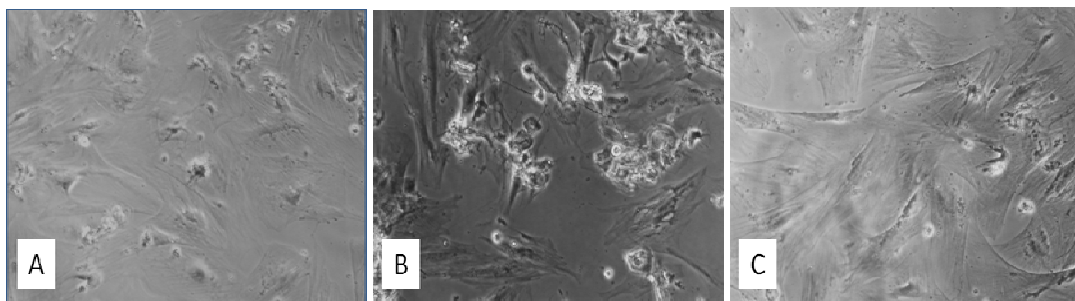


Figure 4.16 Rat Mesenchymal stem cells (MSC) morphology (10 x, 12 h treatment, after 48 h): (A) Negative control MSC, no polymer added; (B) MSC cells with 2 μ L Liopfectamine2000[®] and 1 μ g DNA added; (C) MSC cells with 1,000 μ g 2-Arg-6E-Cl and 1 μ g DNA added.

Although the 2 controls (Lipofectamine2000[®] and PEI) required lower dosages than Arg-PEEAs to reach optimum transfection efficiency, they still showed a significantly higher cytotoxicity than Arg-PEEAs. Since Arg-PEEA had a lower positive charge density than the 2 control transfection reagents, a larger dose of Arg-PEEA was needed to achieve efficient transfection.

The cytotoxicity of Arg-PEEA/DNA complex can also be confirmed by observing cell morphology under light microscope as shown in Figures 4.15 and 4.16 in addition to MTT assay. Figures 4.15 and 4.16 showed the images of HUVEC primary cells and MSC stem cells 48h after treatment of different Arg-PEEA/DNA complexes for 12 h. It can be seen that the cells treated by Arg-PEEA/DNA displayed normal HUVEC (Figure 4.15C) and MSC (Figure 4.16C) morphology, confirming the nontoxic nature of these Arg-PEEAs. In contrast, those HUVEC primary cells and SMC stem cells transfected with Lipofectamine2000[®] (Figure 4.15B and 4.16B) appeared to be somewhat unhealthy. Thus, we can conclude that these newly developed Arg-PEEAs are non-toxic and very safe to a variety of different cell types.

4.E Conclusion

In this work, we tested the hypothesis that the flexibility of the polymers could affect their plasma DNA condensation capability and hence affect their transfection efficiency. A series of water soluble, biocompatible and biodegradable L-Arginine and oligoethylene glycol based poly (ester amide)s (Arg-PEEAs) was prepared and studied for their feasibility as a gene delivery vehicle and cytotoxicity for a variety of cell types, from cell lines to primary cells and stem cells. The relationship of Arg-PEEA polymer structure-function was investigated in terms of the number of methylene and ethylene glycol units. Through various assays and methods, we confirmed that Arg-PEEAs could condense the DNA and form stable complex easily. Some Arg-PEEAs showed better transfection efficiency than Lipofectamine2000[®], and achieved better transfection at a much lower cytotoxicity. The polymer structure-property relationship was revealed and correlated to the transfection performance. This new Arg-PEEA family showed a great potential as a better and safer non-viral transfection agent. Their ability to deliver therapeutic DNAs could be further improved by modifying the polymer structure and transfection protocol.

REFERENCE

1. Anderson, W. F., Human Gene-Therapy. *Science (New York, N.Y.)* **1992**, 256, (5058), 808-813.
2. Luo, D.; Saltzman, W. M., Synthetic DNA delivery systems. *Nature biotechnology* **2000**, 18, (1), 33-37.
3. Mulligan, R. C., The Basic Science of Gene-Therapy. *Science (New York, N.Y.)* **1993**, 260, (5110), 926-932.
4. Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S., Design and development of polymers for gene delivery. *Nature Reviews Drug Discovery* **2005**, 4, (7), 581-593.
5. Wolff, J. A.; Lederberg, J., An Early History of Gene-Transfer and Therapy. *Human Gene Therapy* **1994**, 5, (4), 469-480.
6. Yamanouchi, D.; Wu, J.; Lazar, A. N.; Craig Kent, K.; Chu, C.-C.; Liu, B., Biodegradable arginine-based poly(ester-amide)s as non-viral gene delivery reagents. *Biomaterials* **2008**, 29, (22), 3269-3277.
7. Putnam, D.; Gentry, C. A.; Pack, D. W.; Langer, R., Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, 98, (3), 1200-+.
8. Lynn, D. M.; Anderson, D. G.; Putnam, D.; Langer, R., Accelerated discovery of synthetic transfection vectors: Parallel synthesis and screening of degradable polymer library. *Journal of the American Chemical Society* **2001**, 123, (33), 8155-8156.
9. Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P., A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* **1995**, 92, (16), 7297-301.

10. Verma, I. M.; Somia, N., Gene therapy - promises, problems and prospects. *Nature* **1997**, 389, (6648), 239-242.
11. Zauner, W.; Ogris, M.; Wagner, E., Polylysine-based transfection systems utilizing receptor-mediated delivery. *Advanced Drug Delivery Reviews* **1998**, 30, (1-3), 97-113.
12. Tekade, R. K.; Kumar, P. V.; Jain, N. K., Dendrimers in Oncology: An Expanding Horizon. *Chemical Reviews (Washington, DC, United States)* **2009**, 109, (1), 49-87.
13. Tomalia, D. A.; Naylor, A. M.; Goddard, W. A., Starburst Dendrimers - Molecular-Level Control of Size, Shape, Surface-Chemistry, Topology, and Flexibility from Atoms to Macroscopic Matter. *Angewandte Chemie-International Edition in English* **1990**, 29, (2), 138-175.
14. Wong, K.; Sun, G.; Zhang, X.; Dai, H.; Liu, Y.; He, C.; Leong, K. W., PEI-g-chitosan, a Novel Gene Delivery System with Transfection Efficiency Comparable to Polyethylenimine in Vitro and after Liver Administration in Vivo. *Bioconjugate Chemistry* **2006**, 17, (1), 152-158.
15. Wu, J.; Wang, X.; Keum, J. K.; Zhou, H.; Gelfer, M.; Avila-Orta, C.-A.; Pan, H.; Chen, W.; Chiao, S.-M.; Hsiao, B. S.; Chu, B., Water soluble complexes of chitosan-g-MPEG and hyaluronic acid. *Journal of Biomedical Materials Research, Part A* **2007**, 80A, (4), 800-812.
16. Midoux, P.; Monsigny, M., Efficient gene transfer by histidylated polylysine pDNA complexes. *Bioconjugate Chemistry* **1999**, 10, (3), 406-411.
17. Pack, D. W.; Putnam, D.; Langer, R., Design of imidazole-containing endosomolytic biopolymers for gene delivery. *Biotechnology and Bioengineering* **2000**, 67, (2), 217-223.

18. Thomas, J. L.; Tirrell, D. A., Polyelectrolyte-Sensitized Phospholipid-Vesicles. *Accounts of Chemical Research* **1992**, 25, (8), 336-342.
19. Thomas, J. L.; Barton, S. W.; Tirrell, D. A., Membrane Solubilization by a Hydrophobic Polyelectrolyte - Surface-Activity and Membrane-Binding. *Biophysical Journal* **1994**, 67, (3), 1101-1106.
20. Lim, Y. B.; Kim, C. H.; Kim, K.; Kim, S. W.; Park, J. S., Development of a safe gene delivery system using biodegradable polymer, poly[alpha-(4-aminobutyl)-L-glycolic acid]. *Journal of the American Chemical Society* **2000**, 122, (27), 6524-6525.
21. Lynn, D. M.; Langer, R., Degradable poly(beta-amino esters): Synthesis, characterization, and self-assembly with plasmid DNA. *Journal of the American Chemical Society* **2000**, 122, (44), 10761-10768.
22. Anderson, D. G.; Akinc, A.; Hossain, N.; Langer, R., Structure/property studies of polymeric gene delivery using a library of poly(beta -amino esters). *Molecular Therapy* **2005**, 11, (3), 426-434.
23. Chen, D. J.; Majors, B. S.; Zelikin, A.; Putnam, D., Structure-function relationships of gene delivery vectors in a limited polycation library. *Journal of Controlled Release* **2005**, 103, (1), 273-283.
24. Forrest, M. L.; Koerber, J. T.; Pack, D. W., A Degradable Polyethylenimine Derivative with Low Toxicity for Highly Efficient Gene Delivery. *Bioconjugate Chemistry* **2003**, 14, (5), 934-940.
25. Green, J. J.; Langer, R.; Anderson, D. G., A Combinatorial Polymer Library Approach Yields Insight into Nonviral Gene Delivery. *Accounts of Chemical Research* **2008**, 41, (6), 749-759.
26. Green, J. J.; Zugates, G. T.; Tedford, N. C.; Huang, Y.-H.; Griffith, L. G.; Lauffenburger, D. A.; Sawicki, J. A.; Langer, R.; Anderson, D. G., Combinatorial

modification of degradable polymers enables transfection of human cells comparable to adenovirus. *Advanced Materials (Weinheim, Germany)* **2007**, 19, (19), 2836-2842.

27. Reineke, T. M.; Davis, M. E., Structural Effects of Carbohydrate-Containing Polycations on Gene Delivery. 1. Carbohydrate Size and Its Distance from Charge Centers. *Bioconjugate Chemistry* **2003**, 14, (1), 247-254.

28. Zhu, L.; Lu, Y.; Miller, D. D.; Mahato, R. I., Structural and Formulation Factors Influencing Pyridinium Lipid-Based Gene Transfer. *Bioconjugate Chemistry* **2008**, 19, (12), 2499-2512.

29. Banerjee, P.; Weissleder, R.; Bogdanov, A., Jr., Linear Polyethyleneimine Grafted to a Hyperbranched Poly(ethylene glycol)-like Core: A Copolymer for Gene Delivery. *Bioconjugate Chemistry* **2006**, 17, (1), 125-131.

30. Burke, R. S.; Pun, S. H., Extracellular Barriers to in Vivo PEI and PEGylated PEI Polyplex-Mediated Gene Delivery to the Liver. *Bioconjugate Chemistry* **2008**, 19, (3), 693-704.

31. Fukushima, S.; Miyata, K.; Nishiyama, N.; Kanayama, N.; Yamasaki, Y.; Kataoka, K., PEGylated Polyplex Micelles from Triblock Cationomers with Spatially Ordered Layering of Condensed pDNA and Buffering Units for Enhanced Intracellular Gene Delivery. *Journal of the American Chemical Society* **2005**, 127, (9), 2810-2811.

32. Wong, S. Y.; Sood, N.; Putnam, D., Combinatorial Evaluation of Cations, pH-sensitive and Hydrophobic Moieties for Polymeric Vector Design. *Molecular Therapy* **2009**, 17, (3), 480-490.

33. Futaki, S.; Ohashi, W.; Suzuki, T.; Niwa, M.; Tanaka, S.; Ueda, K.; Harashima, H.; Sugiura, Y., Stearylated arginine-rich peptides: A new class of transfection systems. *Bioconjugate Chemistry* **2001**, 12, (6), 1005-1011.

34. Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B., Polyarginine enters cells more efficiently than other polycationic homopolymers. *Journal of Peptide Research* **2000**, 56, (5), 318-325.
35. Holowka Eric, P.; Sun Victor, Z.; Kamei Daniel, T.; Deming Timothy, J., Polyarginine segments in block copolypeptides drive both vesicular assembly and intracellular delivery. *Nat Mater* **2007**, 6, (1), 52-7.
36. Zhong, Z.; Feijen, J.; Lok, M. C.; Hennink, W. E.; Christensen, L. V.; Yockman, J. W.; Kim, Y.-H.; Kim, S. W., Low molecular weight linear polyethylenimine-b-poly(ethylene glycol)-b-polyethylenimine triblock copolymers: synthesis, characterization, and in vitro gene transfer properties. *Biomacromolecules* **2005**, 6, (6), 3440-3448.
37. Choi, J. S.; Lee, E. J.; Choi, Y. H.; Jeong, Y. J.; Park, J. S., Poly(ethylene glycol)-block-poly(L-lysine) Dendrimer: Novel Linear Polymer/Dendrimer Block Copolymer Forming a Spherical Water-Soluble Polyionic Complex with DNA. *Bioconjugate Chemistry* **1999**, 10, (1), 62-65.
38. Guo, K.; Chu, C. C., Copolymers of unsaturated and saturated poly(ether ester amide)s: synthesis, characterization, and biodegradation. *Journal of Applied Polymer Science* **2008**, 110, (3), 1858-1869.
39. Katsarava, R.; Beridze, V.; Arabuli, N.; Kharadze, D.; Chu, C. C.; Won, C. Y., Amino acid-based bioanalogous polymers. synthesis, and study of regular poly(ester amide)s based on bis(alpha -amino acid) alpha ,w-alkylene diesters, and aliphatic dicarboxylic acids. *Journal of Polymer Science, Part A: Polymer Chemistry* **1999**, 37, (4), 391-407.
40. Chu, C.-C., Biodegradable polymeric biomaterials: an updated overview. *Biomaterials* **2007**, 6/1-6/22.

41. Guo, K.; Chu, C. C., Synthesis, characterization, and biodegradation of copolymers of unsaturated and saturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2007**, 45, (9), 1595-1606.
42. Guo, K.; Chu, C. C., Biodegradation of unsaturated poly(ester-amide)s and their hydrogels. *Biomaterials* **2007**, 28, (22), 3284-3294.
43. Guo, K.; Chu, C. C., Controlled release of paclitaxel from biodegradable unsaturated poly(ester amide)s/poly(ethylene glycol) diacrylate hydrogels. *Journal of Biomaterials Science, Polymer Edition* **2007**, 18, (5), 489-504.
44. Guo, K.; Chu, C. C., Biodegradable and injectable paclitaxel-loaded poly(ester amide)s micropheres: fabrication and characterization. *Journal of Biomedical Materials Research, Part B: Applied Biomaterials* **2009**, 89B, (2), 491-500.
45. Guo, K.; Chu, C. C.; Chkhaidze, E.; Katsarava, R., Synthesis and characterization of novel biodegradable unsaturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2005**, 43, (7), 1463-1477.
46. Huang, S. J.; Bansleben, D. A.; Knox, J. R., Biodegradable polymers: Chymotrypsin degradation of a low molecular weight poly(ester-urea) containing phenylalanine. *Journal of Applied Polymer Science* **1979**, 23, (2), 429-37.

CHAPTER 5

ARGININE-BASED POLY (ESTER AMIDE)S AND PLURONIC DIACRYLATE
CATIONIC HYBRID HYDROGEL FOR TISSUE ENGINEERING AND DRUG
DELIVERY APPLICATIONS

Jun Wu[†], Cynthia Reinhart-King[†] and Chih-Chang Chu^{†, ‡}

[†] Biomedical Engineering Department, Cornell University, Ithaca, New York 14853-4401

[‡]Department of Fiber Science and Apparel Design, Cornell University, Ithaca, New York 14853-4401

5.A Abstract

Hydrogels are very important biomaterial forms for many clinical and pharmaceutical applications. Hydrogels fabricated from a photocrosslinking means have shown particular promising applications due to the ease of fabrication. In this study, we reported a new family of biodegradable and biocompatible cationic hybrid hydrogels synthesized in an aqueous solution via UV- photocrosslinking of two water soluble precursors: arginine-based unsaturated poly(ester amide) (Arg-UPEA) and Pluronic diacrylate (Pluronic-DA). The pH-sensitive Arg-UPEA was prepared via solution polycondensation method and the temperature-sensitive Pluronic-DA was prepared by reacting acryloyl chloride with the end hydroxyl groups of Pluronic. Both precursors have been proved to be non-toxic to cells via MTT assay. The gel fraction (G_f), equilibrium swelling ratio (Q_{eq}), compressive modulus and interior morphology of the hybrid hydrogels were fully investigated. It was found that the incorporation of Arg-UPEA into Pluronic-DA hydrogels significantly changed their Q_{eq} , mechanical strength and interior morphology. The effect of weight feed ratio of these two precursors on the hybrid hydrogels were investigated, and the structure-function relationship of the hybrid hydrogel was studied in terms of the number of methylene groups in Arg-UPEA repeating unit (y in diol segment). The results indicated that increasing methylene groups in the Arg-UPEA repeating unit increased the equilibrium swelling ratio (Q_{eq}) and decreased the compressive modulus of the hydrogels. The cationic nature of the Arg-UPEAs component in the hybrid hydrogels greatly improved the attachment and proliferation of fibroblast cells when comparing with pure Pluronic hydrogel. The bovine aortic endothelial cells (BAEC) viability test in the interior of the hydrogels showed that the positively charged hybrid hydrogel could significantly improve the endothelial cell viability compared with the pure

Pluronic-DA hydrogel. The controlled release of hydralazine (Apresoline) via the hybrid hydrogel system was investigated by HPLC and LC-MS methods. The excellent biocompatibility of these hybrid hydrogels and release profile of hydralazine showed that the hybrid cationic hydrogels developed in this study would have the potential for wound healing and other biomedical applications.

5.B Introduction

Hydrogels refer to certain materials that are able to swell and hold large amounts of water in the wet state¹⁻⁴. Hydrogels generally consist of three-dimensional polymer networks that are cross-linked chemically and/or physically. Because of their significant water contents, hydrogels also possess a degree of flexibility and softness similar to natural tissues¹⁻⁴. In recent years, hydrogels have attracted many interests from clinicians, scientists and engineers in the field of tissue engineering and controlled drug delivery because of their biocompatibility, high water content, 3D microporous structure, permeability for oxygen and nutrients, and tissue-like elastic properties¹⁻⁴.

For drug delivery applications, certain hydrogels could have stimuli-responsive drug release capability (for examples, pH sensitive hydrogel) synchronized with enzymatic or hydrolytic degradation^{3, 5}. One of the very important advantages of hydrogels as drug carriers is that hydrogels could significantly increase the stability of many types of protein/nucleic acid drugs¹. For many tissue engineering applications, it is often desirable to encapsulate cells directly inside hydrogels^{2, 6, 7}. For medical device applications, the most promising applications may involve lens, muscle models, and artificial cartilage^{2, 6, 7}. Hydrogels can also be integrated with microdevices for

various applications including biosensors and diagnostic imaging using photolithographic or other approaches^{1, 8, 9}.

Although natural hydrogels fabricated by physical methods have been widely utilized for biomedical and clinical applications, chemically crosslinked hydrogels offer better control over the structure, physical and mechanical properties^{2, 5, 10}. These hydrogels are fabricated by crosslinking precursors having difunctional/multifunctional groups, such as poly (ethylene glycol) diacrylate (PEG-DA), one of the most used in biomedical field^{2, 3, 11}. Depending on the chemical structure of the precursors, the resulting hydrogels may be responsive to pH, temperature, solvents, and other environmental factors^{2, 3, 11}. By controlling those environmental factors, the hydrogel volume and other related properties could also change and be utilized for varieties of biomedical applications^{2, 3, 11}.

Among the chemical approaches, photo and thermal crosslinking have been widely used to prepare hydrogels. Compared with other approaches, photocrosslinking method has the following advantages^{2, 12}: First, it allows for better spatial and temporal control over the reaction. Second, it will have more rapid entrapment of cells with minimal cell death due to fast curing rates ranging from less than a second to a few minutes. Third, some gelation reactions can be performed under very mild conditions, such as aqueous solution, room temperature, body pH, and even *in situ* in a minimally invasive manner. Above all, photocrosslinking is the preferred approach for preparation of chemically crosslinked hydrogels^{2, 12}.

Among the commercial precursors reported in the literature, Pluronic, also known as [poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide)] tri-block

copolymers] (PEO-PPO-PEO), is one of the most common because it dissolves in cold water, forming a viscous solution and self-associate to form micelles in a diluted aqueous solution¹³⁻¹⁷. At high concentrations above ca. 20 % (w/v), they exhibit a temperature-dependent sol-gel transition behavior¹³⁻¹⁷. The thermo-sensitive properties of Pluronic hydrogels can be attributed to lower critical solution temperature (LCST) behavior that is mainly caused by a delicate balance between hydrophilic and hydrophobic moieties of the polymer. Pluronic derivatives with suitable end functional groups were shown to retain their LCST behavior¹³⁻¹⁷. For biotechnology area, Pluronic is one of the widely used biocompatible polymers with excellent water solubility, low toxicity and immunogenicity¹³⁻¹⁷. Based on that, Pluronic-based hydrogels are also widely used systems for biomedical applications¹³⁻¹⁷.

In this study, we report a new hybrid hydrogel family fabricated from Pluronic and a new cationic and water soluble precursor synthesized from the amino acid-based biodegradable poly(ester amides) (AA-PEAs). Hybrid hydrogels refer to hydrogel systems that contain two or more components of distinct classes of molecules. One of the main purposes of a hybrid hydrogel is to bring new properties/functionalities, such as charge property, hydrophobic/hydrophilic property and functional groups, to the current hydrogel systems by introducing a second precursor component.

AA-PEAs are a newly developed biodegradable biomaterial family that have shown very low cytotoxicity and inflammatory response property, and support natural wound healing. In recent years, AA-PEAs have been widely tested for biomedical applications, such as controlled drug and protein delivery, non-viral gene delivery, and tissue engineering scaffolds¹⁸⁻²⁸. Although unsaturated AA-PEAs have been reported as the precursors to fabricate hybrid hydrogels via photo-means^{21, 22}, these Phe-based

AA-PEA precursors are organic solvent soluble only. A new type of water soluble unsaturated AA-PEAs that are based on L-arginine (Arg) (Arg-UPEA) has been prepared to overcome the water solubility problem of the Phe-based AA-PEA precursors as shown in Figure 5.1. Arg-UPEAs precursor could bring pH sensitivity and positive charge to the resulting hybrid hydrogels.

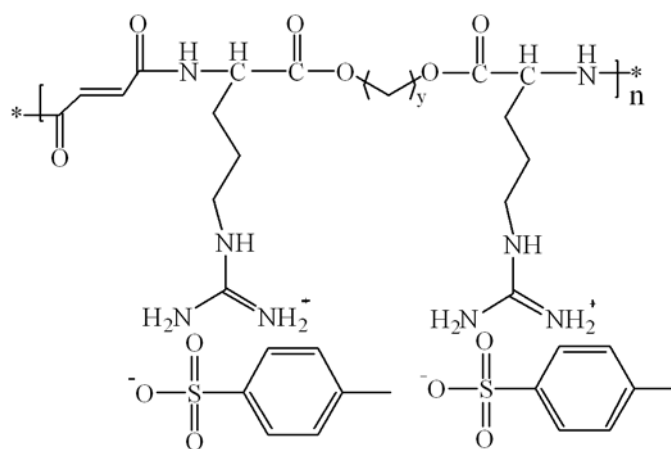


Figure 5.1 Chemical Structure of Unsaturated Arg-PEA (Arg-UPEA)

In this study, a series of novel biodegradable hybrid hydrogels were fabricated from four types of Arg-UPEAs and Pluronic-DA (F127-DA was selected for this study, Figure 5.3) by UV photocrosslinking. The newly synthesized biodegradable Arg-UPEA/Pluronic-DA hydrogels were characterized by gel fraction (G_f), equilibrium swelling ratio (Q_{eq}), compressive modulus and interior morphology. The effects of the precursor's feed ratio (Arg-UPEA to Pluronic-DA) and the type of Arg-UPEA precursor on the property of the hybrid hydrogels were studied. By varying the weight feed ratio of Arg-UPEA to Pluronic-DA, we were able to tune the swelling ratio, mechanical property and pore sizes of the resulting hybrid hydrogels. MTT assay, live-dead assay, cell attachment assay and cell proliferation assay were

conducted to evaluate the cytotoxicity and cellular response of precursors and the resulting Arg-UPEA/Pluronic-DA hydrogels. Our results indicated that Arg-UPEA were nontoxic to the cells even at large dosage treatment. The Arg-UPEA/Pluronic-DA hybrid hydrogels showed a significant improvement of cell attachment, proliferation and viability when compared with pure Pluronic-DA hydrogel. These Arg-UPEA/Pluronic-DA hydrogels were also tested for controlled release of hydralazine shown in Figure 5.2.

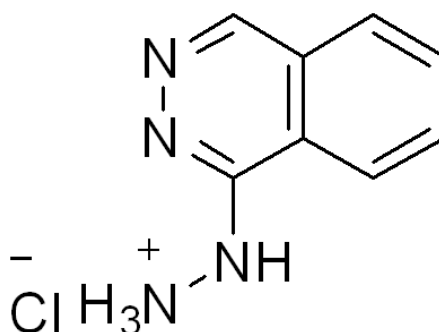


Figure 5.2 Chemical Structure of Hydralazine

5.C Experimental

5.C.1 Materials

Pluronic (F127, MW 12,600 and 70% PEG content) and acryloyl chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Triethylamine from Fisher Scientific (Fairlawn, NJ) was dried via refluxing with calcium hydride and then distilled before use. 2-Hydroxy-1- [4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959) was donated by Ciba Specialty Chemicals Corporation. L-Arginine (L-Arg), *p*-toluenesulfonic acid monohydrate, fumaryl chloride, ethylene glycol, 1,3-propanediol, 1, 4-butanediol, 1,6-

hexanol and *p*-nitrophenol were all purchased from Alfa Aesar (Ward Hill, MA) and used without further purification. 3- (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) purchased from Sigma (St. Louis, MO) was used to evaluate the cell toxicity of Arg-UPEA and Arg-UPEA/Pluronic-DA hydrogel. Hydralazine chlorine salt used for controlled release test was also purchased from Sigma (St. Louis, MO). Other chemicals and reagents if not otherwise specified were purchased from Sigma (St. Louis, MO).

5.C.2 Synthesis of Hydrogel Precursors

5.C.2.a Synthesis of Pluronic-Diacrylate (Pluronic-DA)

Pluronic-DA (F127-DA) was synthesized according to a modified procedure based on a previously reported method¹⁶ (Figure 5.3). In brief, 2.0 mmol of F127 was dissolved in 150 mL of benzene and heated to 45 °C with stirring until a complete dissolution. After the solution was cooled to room temperature, 1.67 mL (12.0 mmol) of triethylamine, at a threefold molar excess concentration (based on the mole amount of F127 diol end groups), was added to the F127 solution. Then, 0.97 mL (12.0 mmol) of acryloyl chloride, also at a threefold molar excess concentration, was dissolved in 50 mL benzene solution and added dropwise to the F127 solution through a dropping funnel. The mixture was stirred and cooled to 0 °C for 12 h with nitrogen protection. After that, the reaction was stopped and the insoluble triethylamine salt was removed by filtration. The F127-DA product was then precipitated out by pouring the reaction solution into 800 mL cold hexane. The F127-DA precipitate was collected and purified by filtration, re-dissolved in 50 mL of benzene, and precipitated in 500 mL of cold hexane, then repeated the above steps twice. The F127-DA polymer was finally

dried for 24 h in a vacuum oven at 25 °C and the dried product was sealed and stored in the refrigerator (approximately 4 °C) for future use.

5.C.2.b Synthesis of Unsaturated Arginine Poly (ester amide) (Arg-UPEA)

The unsaturated Arg-based poly (ester amide) s (Arg-UPEAs) were synthesized by the same procedures reported before^{19, 25, 28}. Briefly, the synthesis could be divided into the following three major steps: the preparation of unsaturated di-*p*-nitrophenyl ester of dicarboxylic acid (**I**) (Figure 5.4); the preparation of tetra-*p*-toluenesulfonic acid salts of bis (L-arginine), α , ω -alkylene diesters (**II**) (Figure 5.5); and the synthesis of Arg-UPEAs (**III**) via solution polycondensation of (**I**) and (**II**) (Figure 5.6)

Unsaturated di-*p*-nitrophenyl esters of dicarboxylic acid (Monomer **I**), di-*p*-Nitrophenyl Fumarate (**NF**), was prepared by reacting fumaryl chloride with *p*-nitrophenol as previously reported²⁵. Four types of *p*-toluenesulfonic acid salt of L-arginine diesters (Monomer **II**) were prepared in this study: tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) ethane diesters **Arg-2-S** (y=2); tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) propane diesters, **Arg-3-S** (y=3); tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) butane diesters, **Arg-4-S** (y=4); tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) hexane diesters, **Arg-6-S** (y=6).

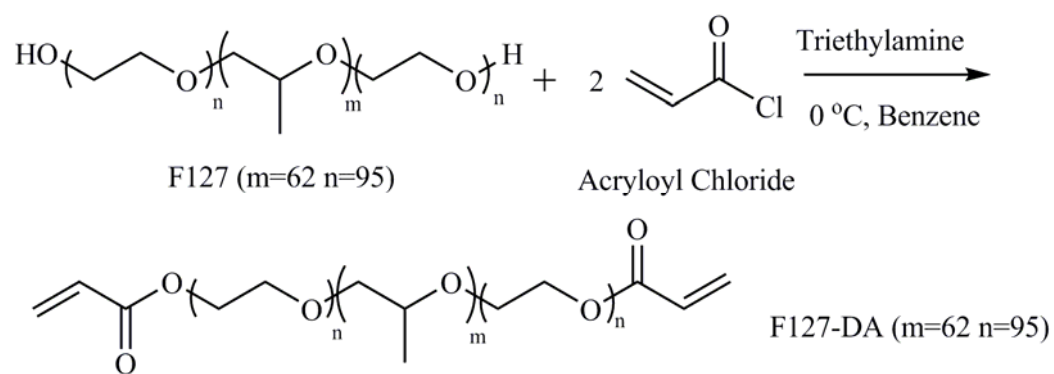


Figure 5.3 Synthesis of Pluronic-DA

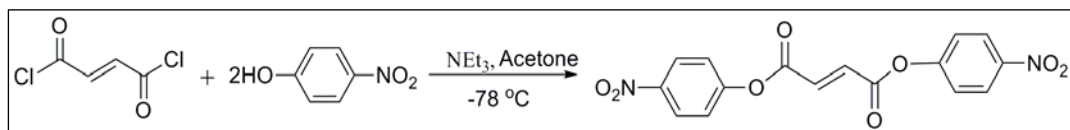


Figure 5.4 Synthesis of Monomer **I**, Di-*p*-nitrophenyl Ester of Dicarboxylic Acids

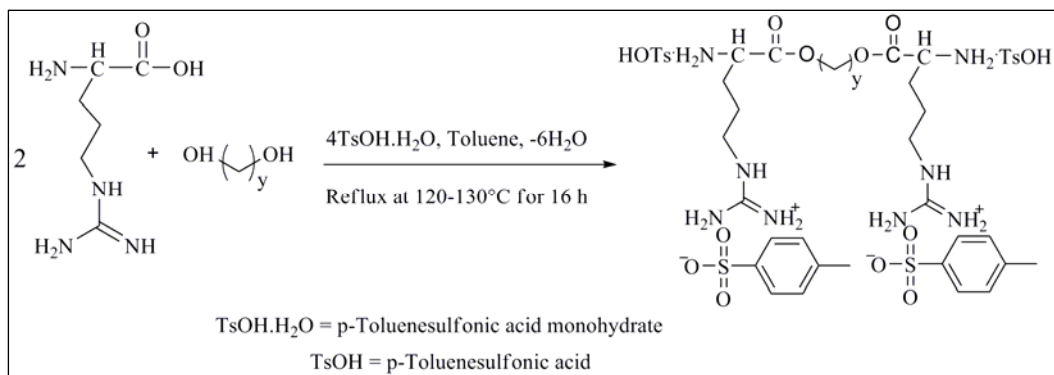


Figure 5.5 Synthesis of Monomer **II**, Tetra-*p*-toluenesulfonic Acid Salt of Bis (L-arginine) Alkylene Diesters

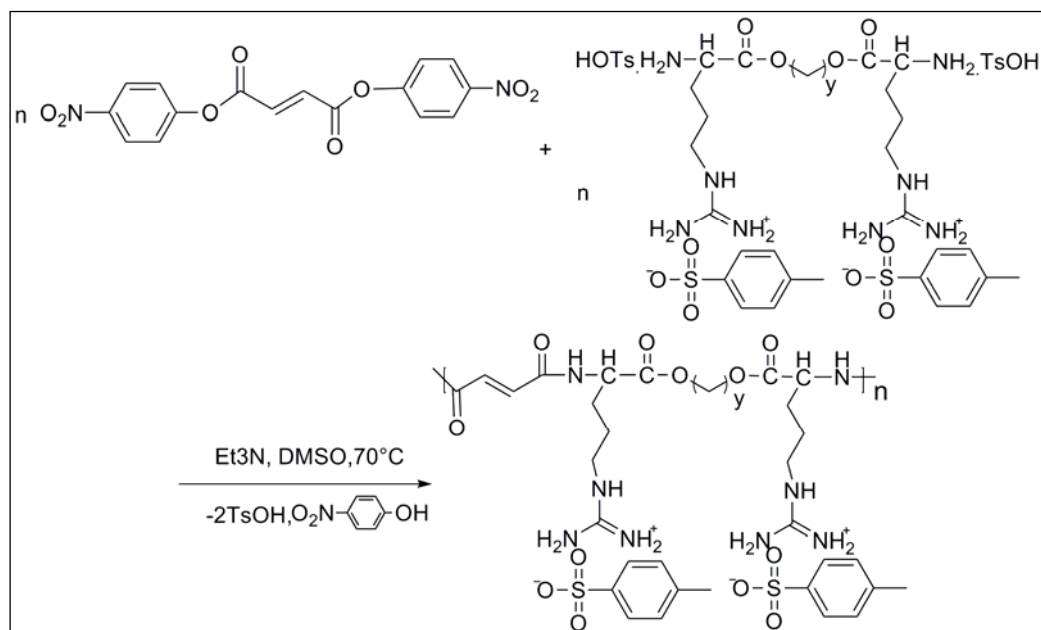


Figure 5.6 Synthesis of Arg-UPEAs from monomers I and II

Arg-UPEAs were prepared by the solution polycondensation of **(I)** and **(II)** monomers (**NF and Arg-2-S, Arg-3-S, Arg-4-S, Arg-6-S**) at different combinations. The Arg-UPEAs are labeled as x-UArg-y-S, where x and y are the number of CH and CH₂ groups in diacid and diol segments, respectively, and U means the Arg-PEA is unsaturated. In this report, x was fixed at 2 because only one type of monomer **I (NF)** was used. An example of the synthesis of 2-UArg-6-S via a solution polycondensation is given here. Monomers NF (1.0 mmol) and Arg-6-S (1.0 mmol) in 2.0 mL of dry DMSO were mixed well by vortexing. The mixture solution was heated up to 75 °C with stirring to obtain a uniformed mixture. Triethylamine (0.31 mL, 2.2 mmol) was added drop by drop to the mixture at 75 °C with vigorous stirring until the complete dissolution of the monomers. The solution color turned into yellow after several minutes. The reaction vial was then kept for 48 hrs at 75 °C in a thermostat oven without stirring. The resulting solution was precipitated in cold ethyl acetate, decanted, dried, re-dissolved in methanol and re-precipitate in cold ethyl acetate for a further purification of 3 times before drying under *vacuum* at room temperature. The final Arg-UPEAs are yellow or pale yellow solid powder. The Arg-UPEAs synthesized with different combinations of diacids and diols building blocks are: 2-UArg-2-S, 2-UArg-3-S, 2-UArg-4-S and 2-UArg-6-S.

5.C.3 Fabrication of Arg-UPEA/Pluronic-DA Hybrid Hydrogels

Arg-UPEA/Pluronic-DA hybrid hydrogels were prepared by the photopolymerization of two precursors (Arg-UPEA and Pluronic-DA) at different weight ratios in water with an initiator. The Arg-UPEA and Pluronic-DA precursors were purified first by dissolving the precursors in distilled water and were dialyzed against deionized water (MW cut off 4,000) for 2 days. After that, the solutions were

lyophilized for 3 days using a Virtis Freeze Drier (Gardiner, NY) under vacuum at -48 °C. An example of the fabrication of a hybrid hydrogel was given below: 0.08 g of 2-UArg-2-S and 0.32 g F127-DA (1/4 weight ratio of 2-UArg-2-S/F127-DA) were added into a glass vial and dissolved in 2.0 mL of deionized water to form a clear homogeneous solution with light yellow color, which was brought by Arg-UPEA. 0.004 g of photoinitiator Irgacure 2959 (1 wt% of total amount of precursors) was added into the precursors' solution and dissolved completely. The hydrogel precursor solution was transferred to a custom-made 20 well Teflon mold (diameter 12 mm and thickness \approx 4 mm for each well) using a micropipette. The precursor solution in the molds was irradiated by a long-wavelength UV lamp (365 nm, 100 W) for specified time (15 min) at room temperature. The irradiation distance is 5-10 cm. The resultant hydrogels were moved from the mold and immersed in distilled water at room temperature for 48 hr to remove any residual chemicals. The distilled water was replaced periodically. After this purification process, the hydrogel was soaked in distilled water to reach swelling equilibrium, and dried in vacuum at room temperature for 48 hr before further characterization and application.

The gel fraction (G_f) of the resulting hydrogels was calculated by the following equation:

$$G_f = (W_d / W_p) \times 100 \%$$

where W_d is the weight of dry hydrogel and W_p is the total weight of the two precursors and the photoinitiator.

5.C.4 Measurements

The physicochemical properties of the monomers, polymers and hydrogels were characterized by various standard methods. For Fourier transform infrared (FTIR)

characterization, the dried samples were ground into powders and mixed with KBr at a sample/KBr ratio of 1:10 (w/w). FTIR spectra were then obtained with a PerkinElmer (Madison, WI) Nicolet Magana 560 FTIR spectrometer with Omnic software for data acquisition and analysis. ^1H NMR spectra were recorded with a Varian Unity Inova 400-MHz spectrometer (Palo Alto, CA). Deuterated water ($\text{D}_2\text{O}-d_2$) or deuterated dimethyl sulfoxide ($\text{DMSO}-d_6$) (Cambridge Isotope Laboratories, Andover, MA) with tetramethylsilane as an internal standard was used as the solvent. MestReNova software was used for the data analysis. Elemental analyses of the polymers/hydrogels were performed with a PE 2400 CHN elemental analyzer by Atlantic Microlab (Norcross, GA). The thermal property of the synthesized precursors (Arg-UPEAs and Pluronic) was characterized with a DSC 2920 (TA Instruments, New Castle, DE). The measurements were carried out from -10 to 200 °C at a heating rate of 10 °C/min and at a nitrogen gas flow rate of 25 mL/min. TA Universal Analysis software was used for thermal data analysis. The solubility of Arg-UPEAs in common organic solvents at room temperature was assessed by using 1.0 mg/mL as solubility criteria. The quantitative solubility of Arg-UPEAs in distilled water at room temperature was also measured by adding distilled water to the polymer sample step by step until the clear solution was obtained. Slight heating was need for some types of Arg-UPEAs.

For the molecular weight measurement, Arg-UPEAs were prepared at a concentration of 1 mg/mL in a 0.1 % (w/v) LiCl in DMAc solution. The sample molecular weights were determined from a standard curve generated from polystyrene standards with molecular weights ranging from 841.7 kDa to 2.93 kDa that were chromatographed under the same conditions as the samples. The standard curve was generated from a 3rd order polynomial fit of the polystyrene standard molecular weights.

Interior morphology of Arg-UPEA/Pluronic-DA hydrogels was investigated by SEM. The swollen hydrogel samples, after reaching their maximum swelling ratio in distilled water at room temperature, were quickly frozen in liquid nitrogen and then freeze-dried under vacuum at -48 °C for 3 days until all water inside the hydrogel was sublimed. The freeze-dried hydrogel samples were then cut and fixed on aluminum stubs and then coated with gold for 30 seconds for interior morphology observation with a scanning electron microscope instrument (Leica S440, Germany).

5.C.5 Hydrogel Swelling Ratio & Swelling Kinetics

The equilibrium swelling ratio (Q_{eq}) of the hydrogel is calculated by the following equation:

$$Q_{eq} = [(W_e - W_d) / W_d] \times 100 \%$$

where W_s is the weight of a swollen hydrogel at equilibrium and W_d is the weight of the corresponding dry hydrogel at $t = 0$.

The swelling kinetics of the Arg-UPEA/F127-DA hydrogels was measured over a period of 4 days at room temperature. Each dry Arg-UPEA/F127-DA gel sample was weighed and immersed in 20 mL of solutions with different parameters for predetermined periods. Before weighing, the samples were taken from the solutions and blotted with filter papers to remove excess surface water. The swelling ratio (Q) of the hydrogel at time t is calculated by the following equation:

$$Q = [(W_t - W_d) / W_d] \times 100 \%$$

where W_t is the weight of swollen hydrogel at time t and W_d is the weight of the dry hydrogel at $t = 0$. All swelling ratio results were obtained from triplicate samples and data were expressed as the means \pm standard deviation.

5.C.6 Compressive Modulus Measurement by Dynamic Mechanical Analyzer (DMA)

The mechanical property of the Arg-UPEA/F127-DA hydrogels was measured by a DMA 2980 Dynamic Mechanical Analyzer (TA Instruments Inc., New Castle, DE) in a “controlled force” mode (CF-mode). The swollen hydrogel samples in circular disc shape were submerged in distilled water and mounted between the movable compression clamp (diameter 30 mm) and the fluid cup with a 0.1 N preloading force. A force ramp from 0.1 N at a rate of 0.3 or 0.5 N/min was applied. All measurements were carried out at room temperature. The compression elastic modulus (E) of the swollen hydrogel was extracted by plotting the compressive stress versus strain. All compression elastic modulus data in this study were obtained from triplicate samples and data were expressed as the means \pm standard deviation.

5.C.7 Cell Culture Study

Bovine endothelial aorta cells (BAECs) were purchased from VEC Technologies, kindly offered by Professor Cynthia Reinhart-King at Department of Biomedical Engineering of Cornell University. BAECs were cultured at 37 °C in 5 % CO₂ in Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10 % Fetal Clone III (HyClone, Logan, UT), and 1 % each of penicillin–streptomycin, MEM amino acids (Invitrogen, Carlsbad, CA), and MEM vitamins (Mediatech, Manassas, VA). BAECs

were used from passages 8–12. Media was changed every 2 days. BAECs were grown to 70 % confluence before splitting or harvesting. Cell culture plates were coated with 2 wt% gelatin aqueous solution before using.

Detroit 539 human fibroblast cells were purchased from ATCC. The fibroblast cells were maintained at 37 °C in 5 % CO₂ in Dulbecco's minimal essential medium (DMEM) supplemented with 10 % FBS (Germini, Woodland, CA) and 1 % each of penicillin–streptomycin, MEM amino acids (Invitrogen, Carlsbad, CA), and 0.1 % lactalbumin hydrolysate. Fibroblast cells were used from passages 10-20. Cell media was changed every 2 days. Cells were grown to 70 % confluence before splitting or harvesting.

5.C.8 Cytotoxicity Evaluation of Precursors by MTT Assay

The cytotoxicity evaluation of the Arg-UPEA and F127-DA precursors was performed by MTT assay. Arg-UPEA or F127-DA aqueous solutions (2 wt% for Arg-UPEA and 10 wt % for F127-DA) were obtained by dissolving the purified polymer in PBS buffer solution. Cultured bovine endothelial aorta cells (BAEC) were seeded cell medium at an appropriate cell density concentration (3,000 cells/well) in 96-well plates and incubated overnight. After 12 h, the BAECs were treated with the freshly prepared aqueous Arg-UPEA or F127-DA PBS solution with various volumes, the BAEC without any polymer were used as the control. After 48 h treatment and incubation, 20 µL of MTT solution (5 mg/mL) was added to each well, followed by 4 h incubation at 37 °C, 5 % CO₂. Then the cell culture medium including polymer solution was carefully removed and 200 µL of acidic isopropyl alcohol (with 0.1 M HCl) was added to dissolve the formed formazan crystal. The plate was slightly

shaken for 20 mins to make sure that the purple crystal dissolved completely. Absorbance (OD) was measured immediately at 570 nm (subtract background reading at 690 nm) using a microplate reader (VersaMax Tunable Microplate reader Molecular Devices, USA). The BAECs viability (%) was calculated according to the following equation:

$$\text{Viability (\%)} = (\text{OD}_{570 \text{ (sample)}} - \text{OD}_{620 \text{ (sample)}}) / (\text{OD}_{570 \text{ (control)}} - \text{OD}_{620 \text{ (control)}}) \times 100\%$$

Where the $\text{OD}_{570 \text{ (control)}}$ represented the measurement from the wells treated with medium only and the $\text{OD}_{570 \text{ (sample)}}$ represented the measurement from the wells treated with various polymer volumes.

5.C.9 Cell Attachment and Proliferation on Arg-UPEA/F127-DA Hybrid Hydrogels Surface

The cell attachment and proliferation on the Arg-UPEA/F127-DA hybrid hydrogel surfaces was evaluated by cell morphology. Pure F127-DA hydrogel was selected as the hydrogel control and the cell culture plate without any treatment was used as the negative control. The cells used for this study were Detroit 539 human fibroblasts. The purified hydrogels were cut into round shape with the diameter that just filled the well of 24-well cell culture plates. Before being put into the 24-well cell culture plates, the hydrogels were sterilized under UV light (from the cell culture hood) for 1 h. After that, the hydrogels were washed twice by PBS buffer and cell culture media. Then, the hydrogels were placed into the wells of the cell culture plate and fixed by sterilized rubber ring which has the same diameter as the well of cell culture plate. Detroit 539 human fibroblasts were seeded at an appropriate cell density (10,000 cells/well) and incubated overnight. After 48 h incubation, the cell attachment and proliferation on the hydrogel surface was record by an optical microscope. For MTT

assay, the attached cells were first detached from the hydrogel surface or cell culture plate surface by trypsin (0.1 mg/mL) treatment, then the detached cells were transferred into a new 96 cell culture place, after 12 h incubation, the MTT assay were processed.

5.C.10 Cell Viability inside Arg-UPEA/F127-DA Hybrid Hydrogels

In order to test the cell viability inside the Arg-UPEA/F127-DA hybrid hydrogels, the BAEC cells were encapsulated into these hydrogels by the following steps: Purified hydrogel precursors and initiators were dissolved in a PBS buffer, and then the cells (10^7 /mL in media), FBS, antibiotics and other nutrients of the complete cell culture media were added. The final mixture solution has 20 wt % precursors, 60,000 cells/mL, and 10 wt % FBS inside. All other components of the complete cell culture media in the mixture have the exactly same concentration as the normal BAEC cell culture media recommended by ATCC. The mixture were injected into 24-well cell culture plate (0.5 mL per well) by a pipette and crosslinked under 100 W UV irradiation for 5 mins and the irradiation distance is 5 cm. After crosslinking, 0.5 mL complete cell culture media was added into each well. The BAEC-loaded hydrogels were incubated for 2 weeks at 37 °C, 5 % CO₂. Cell culture media was changed every other day. The live-dead assay was then performed according to the manufacturer protocol (LIVE/DEAD[®] Cell Viability Assay Kit from Invitrogen).

5.C.11 Controlled Release of Hydralazine (Apresoline) via Arg-UPEA/F127-DA Hybrid Hydrogels

The release of hydralazine from the Arg-UPEA/Pluronic-DA hydrogels were carried out in a PBS buffer at 37 °C. Hydralazine was preloaded into the hydrogel samples (in small pellet form) by two different means: a) hydralazine was directly mixed with Arg-UPEA and Pluronic-DA precursors and photo-initiators in distilled water, and the solution mixture was UV irradiated to form drug-impregnated hydrogels; or b) hydralazine was first mixed with cationic (such as chitosan or Arg-PEA/Arg-UPEA) and anionic (such hyaluronic acid and alginate) polymers; then the above polyelectrolyte-drug complex would mix with precursors/initiators in distilled water, and UV irradiated to form hydrogel. The drug loaded hydrogels from a or b method above were then placed inside small vials containing 10.0 mL PBS solution (one piece of hydrogel per vial). The vial was incubated at 37 °C with a constant reciprocal shaking (ca. 100 rpm). The hydralazine contents were then analyzed by a high performance liquid chromatography (HPLC). To determine the release amounts of the drug at predetermined time points, 50 µL of the immersion solution was removed from the vial and added into a HPLC vial followed by adding 950 µL PBS buffer to dilute the solution. The solution was thoroughly vortexed before tested by a HPLC (HP 1100 model, Palo, Alto, CA, USA) equipped with a diode array detector. A C18 reversed phase column (5 µm, 4.6X250 nm, Alltech Adsorbosphere XL) was used as the stationary phase, while the mobile phase consisted of acetonitrile and water (pH 3.0) in the volume ratio 63:37. The injection volume was 50 or 100 µL based on the released hydralazine concentration and the mobile phase was pumped at a flow rate of 2.0 mL/min. Detection was at 365 nm with a UV detector. ChemStation (Palo, Alto, CA, USA) software was used for data analysis. All drug release tests at each time point were carried out in triplicate and variation was expressed as a standard error of the mean. The confirmation of hydralazine chemical structure was carried out by GC-MS method and the major diagnostic MS fragments were analyzed.

5.C.12 Statistics

Where appropriate, the data are presented as mean \pm standard error of the mean calculated over at least three data points. Significant differences compared to control groups were evaluated by unpaired Student's t-test or Dunnet test at p 0.05, and between more than two groups by Tukey's test with or without one-way ANOVA analysis of variance. JMP software (version 8.0, from SAS Company) was used for data analysis.

5.D Results and Discussions

The goal of this study is to expand the applications of Arg-PEA, especially for the areas of tissue engineering and drug delivery. The previous tests indicated that the Arg-PEAs had excellent water solubility, positive charge and good biocompatibility, which greatly arouse our interests²⁸. However, the reported Arg-PEAs did not have any functional groups for further chemical modifications²⁸, in order to chemically incorporate the Arg-PEA to the scaffolds; double bonds were introduced to the Arg-PEA backbone so that crosslinking method could be applied.

5.D.1 Preparation and Characterization of Precursors

5.D.1.a Synthesis and Characterization of Pluronic-DA

The Pluronic (F127) with an average molecular weight of 12,600 was used here to prepare the precursor for the photo-crosslinking. The synthesis of F127-DA was followed the previous report¹⁶. It was found that the reaction temperature and N₂ protection were important for the successful preparation of F127-DA. The optimized

reaction temperature is 0 °C and the reaction time would need 12-16 h under this temperature. High temperature, such as 60 °C or even higher, would reduce the reaction time to 2-3 h. However, the final product would show some yellow color because of the side reactions. In order to obtain final product with white color, 0 °C, N₂ protection and magnetic bar stirring were suggested for the F127-DA synthesis. The product should be re-purified by dialysis method before any biological applications. The final product was white powder and could dissolve in distilled water to form clear and transparent solution at room temperature or 4 °C. Vigorous stirring/shaking was not suggested for making the solutions because of the production of bubbles. The chemical structure of F127-DA was confirmed by ¹HNMR and FTIR. The FTIR peak of the ester bond is around 1725 cm⁻¹, which indicated the successfully acrylation of Pluronic polymers. To avoid the self-crosslinking, the final product should be vacuum sealed and stored at 4 °C or -20 °C in dark place. And it was suggested that the Pluronic-DA should be used within 3-4 months after preparation.

5.D.1.b Synthesis and Characterization of Arg-UPEAs

The unsaturated di-*p*-nitrophenyl esters of dicarboxylic acids (**NF**) was synthesized and characterized as previous reports (Figure 5.3)²⁵. The tetra-*p*-toluenesulfonic acid salts of bis (L-arginine) alkylene diesters have been recently designed and synthesized²⁸. The amounts of *p*-toluenesulfonic acid used was the main difference of monomer synthesis between the current Arg-based monomers and other hydrophobic amino acid based monomers^{19-23, 25, 28-32}. The excessive *p*-toluenesulfonic acid was needed because of the strong alkalinity of the guanidine group of Arginine. The *p*-toluenesulfonic acid preferred to react with guanidine group first to form a stable salt, then reacted with the amine group of arginine. Four types of monomer **II**

were prepared (figure 5.5): Tetra-*p*-toluenesulfonic acid salt of L-**Arginine** ethane-1,**2**-diester (**Arg-2-S**), Tetra-*p*-toluenesulfonic acid salt of L-**Arginine** propane-1,**3**-diester (**Arg-3-S**), Tetra-*p*-toluenesulfonic acid salt of L-**Arginine** butane-1,**4**-diester (**Arg-4-S**), and Tetra-*p*-toluenesulfonic acid salt of L-**Arginine** hexane-1,**6**-diester (**Arg-6-S**). The only difference among these monomers is the methylene chain length (*y*) in the diol part between the two adjacent ester groups: number of CH₂ varies from 2 to 6 (**Arg-2-S** to **Arg-6-S**). The chemical structures of these 4 types of Arg-based monomer **II** were all confirmed by FTIR and ¹HNMR. The details of characterization data were discussed elsewhere²⁸.

Arg-UPEAs were prepared according to the reaction scheme in figure 5.6. Four types of Arg-UPEAs were prepared with varying *y* values: 2-UArg-2-S, 2-UArg-3-S, 2-UArg-4-S, and 2-UArg-6-S. The yield of final product was high and more than 85 %. The chemical structure of Arg-UPEAs was confirmed by FTIR and HNMR. The yields, water solubility, charge density, glass transition temperature (*T_g*), molecular weight of repeating unit, molecular weight were given in Table 5.1. The Arg-UPEAs were moisture sensitive and should be stored in sealed bottles at 4 °C or lower temperature in dark place.

Table 5.1 Chemical and Physical Properties of Arg-UPEAs

	2-UArg-2-S	2-UArg-3-S	2-UArg-4-S	2-UArg-6-S
Yield (%)	87 %	91 %	93 %	89 %
Charge Density (mol/kg)	2.50	2.46	2.42	2.34
Molecular Weight of Repeating Unit (g/mol)	798.9	812.9	826.9	855.0
Molecular Weight (M_n , kg/mol)	12.93	14.56	15.71	14.82
Molecular Weight (M_w , kg/mol)	14.01	16.14	17.49	16.33
PDI (M_w/M_n)	1.08	1.11	1.11	1.10
T_g (°C)	112	103	94	88
Water Solubility (mg/mL)	20±2	30±2	10±1	2±0.5

For the solubility of Arg-UPEA in common organic solvents, 1.0 mg/mL was used as a standard whether a polymer is soluble or insoluble at room temperature. Due to their strong polar nature, Arg-UPEAs tended to dissolve in polar solvents. All the synthesized Arg-UPEAs were soluble in polar organic solvents like DMSO, DMF and methanol, but did not dissolve in non-polar or weak polar organic solvents like ethyl acetate or chloroform. And the Arg-UPEAs showed decreased water solubility compared to the reported saturated Arg-PEAs. The effect of y material parameters on Arg-UPEA water solubility (Table 5.1) revealed that y had a major impact on the water solubility of Arg-UPEAs; and an increase in the methylene chain length in the diols (y) part reduced the water solubility significantly due to the increasing hydrophobicity. By adjusting the y , the Arg-UPEAs' solubility (hydrophilicity/hydrophobicity) could be fine tuned to meet specific needs.

The molecular weight (M_n and M_w) of 2-UArg-2-S, 2-UArg-3-S, 2-UArg-4-S, 2-UArg-6-S were obtained with the help of MediVas, LLC. The MW data in Table 5.1 indicated that all the Arg-UPEAs had M_n between 12.5 kg/mol and 16.0 kg/mol with narrow polydispersity (PDI) of 1.07 – 1.10. The y values did not have any significant impact on MW and PDI of Arg-UPEAs. Compared with saturated Arg-PEAs, the MW of the Arg-UPEAs did not show any big difference and were in the same range.

For the thermal property of the Arg-UPEAs, they did not have melting points (T_m), which were consistent with the reported saturated Arg-PEAs. The glass transition temperature (T_g) of Arg-UPEAs (Table 5.2) were in the range of 85-115 °C, which had the same range as the T_g of phenylalanine-based unsaturated PEAs²⁵. Due to the presence of double bonds in the repeating unit of Arg-UPEA backbone, much higher T_g values were observed when compared with the saturated Arg-PEAs. For example,

2-UArg-2-S had a T_g 112 °C, while 2-Arg-2-S had a T_g 52 °C, a 100% increase in T_g by simply introducing unsaturated C=C bond in the backbone. Pang et al. reported that the location of C=C bonds may also have a significant effect on T_g ³⁰. For example, if the C=C bonds were located in the side chain, the T_g value may decrease significantly³⁰. Similar magnitude of T_g change was also reported for the Phe based PEAs^{19, 25}. An examination for the effect of the number of methylene groups in the diol (y) part of the Arg-UPEAs revealed that an increase in y led to a lower T_g . The T_g decreased from 112 °C to 88 °C when the y value was increased from 2 to 6. This relationship is consistent with non-ionic hydrophobic amino acid-based and many other PEA systems, such as the saturated Phe-PEAs, for example, if x value was fixed at 4, the T_g decreased from 59 °C to 49 °C when the y value was increased from 4 to 6¹⁹ (4-Phe-4 vs. 4-Phe-6).

5.D.2 Fabrication and Characterization of Arg-UPEA/Pluronic-DA Hybrid Hydrogels

Theoretically, Arg-UPEA should be able to undergo free radical crosslinking using an initiator because of its double bond moiety. In this study, however, we found that Arg-UPEA itself could not form a hydrogel network with good 3-D shape easily even at a high concentration. (e.g. 50.0 wt% solution) The difficulty in self-crosslinking between double bonds could be caused by the side guanidino groups of arginine. Therefore, Pluronic-DA was selected as a second crosslinker to facilitate polymerization of Arg-UPEA, with Irgacure 2959 as the photoinitiator in an aqueous system via the UV photo-polymerization. The initiator was previously reported to cause minimal toxicity (cell death) over a broad range of mammalian cell types and species ranging from human fetal osteoblasts to bovine chondrocytes³³.

All the fabricated Arg-UPEA/F127-DA hydrogels were transparent after reaching their swelling equilibrium. Figure 5 shows an example of the 2-UArg-2-S/F127-DA hydrogel (feed weight ratio: 1/4). The right image was a dried hydrogel and the left was the same hydrogel at a swelling equilibrium. The properties of these hydrogels, such as the swelling behavior, compression modules and interior morphology were systematically examined. And the dependence of these properties on the precursor property and feed ratios of Arg-UPEA to F127-DA were examined as well. The data are summarized in Table 2. The successful fabrication of Arg-UPEA/F127-DA hybrid hydrogels was confirmed by elemental analysis. By measuring the nitrogen element percentage of dried hydrogels, it was confirmed that most of the Arg-UPEA was successfully crosslinked with F127-DA. The measured N contents in Table 2 were slightly higher than the theoretical value (in the parentheses), suggesting that the hybrid hydrogels had Arg-UPEA precursor incorporated since F127-DA didn't have any N content. The gel fraction data (G_f) data showed that pure F127-DA hydrogel has a higher G_f value (more than 90 %) than the Arg-UPEA/F127-DA hybrid hydrogel (around 80 %). The reason for this G_f difference is that F127-DA has higher activity than Arg-UPEA. The low G_f value of Arg-UPEA/F127-DA hybrid hydrogel means more uncrosslinked precursors inside the hydrogel and the purification for the Arg-UPEA/F127-DA hybrid hydrogel need a longer time.

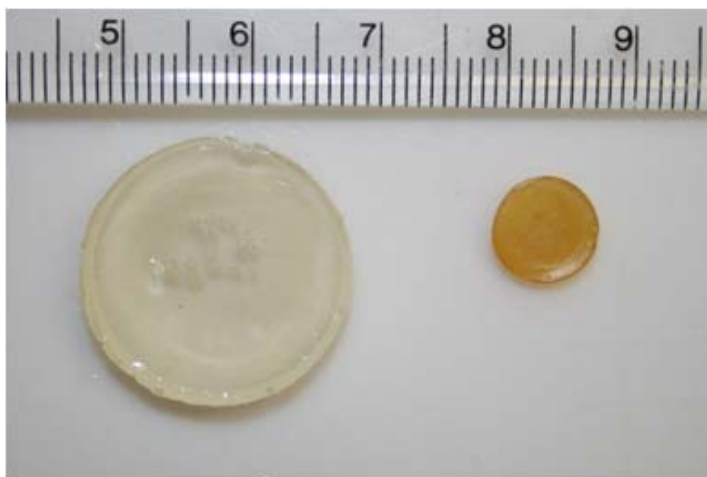


Figure 5.7 Image of hybrid hydrogel of 2-UArg-2-S/F127-DA with a feed ratio of 1: 4; right: dried hydrogel; left: hydrogel at swelling equilibrium

5.D.3 Equilibrated Swelling Ratio & Swelling Kinetics of Arg-UPEA/Pluronic-DA in DI Water and Buffers

The swelling kinetics of the Arg-UPEA/Pluronic-DA hydrogel was studied over a period of 4 days in deionized (DI) water at room temperature. For example, Figure 5.8 showed the hydrogels of varied compositions (different types of Arg-UPEAs, fixed feed weight ratio of Arg-UPEA to F127-DA) had a high swelling rate during the initial 3 hours. From Figure 5.8, for the pure F127-DA hydrogel and hybrid hydrogels, the swelling rate difference among them was not big. After the initial 3 hours, the swelling rate leveled off, and finally reached their swelling equilibrium within about 12-18 hours. The swelling ratios at equilibrium were summarized in Table 5.2.

Table 5.2 Arg-UPEA/F127-DA Hybrid Hydrogels and Their Physicochemical Properties

Sample	Weight Ratio	G _f (%)	Q _{eq} (%)*	Compressive Modulus* (KPa)	N (%)
F127-DA	100:0	92	1144±17	12.27±0.34	0.00 (0.00)
F127-DA/2-UArg-2-S	4:1	83	1785±74	4.85±0.41	2.80 (2.61)
F127-DA/2-UArg-3-S	4:1	80	1843±77	4.43±0.23	2.76 (2.54)
F127-DA/2-UArg-4-S	4:1	81	1917±59	3.77±0.24	2.71 (2.55)
F127-DA/2-UArg-6-S	4:1	79	2158±98	2.58±0.25	2.62 (2.50)
F127-DA/2-UArg-2-S	3:2	77	2014±63	1.97±0.21	5.61 (5.40)
F127-DA/2-UArg-3-S	3:2	79	2191±94	1.91±0.11	5.52 (5.44)
F127-DA/2-U-Arg-4-S	3:2	76	2287±63	1.63±0.19	5.43 (5.27)
F127-DA/2-UArg-6-S	3:2	75	2407±117	0.77±0.17	5.24 (5.10)

*Q_{eq} (%) and compressive modulus was measured in DI water at room temperature.

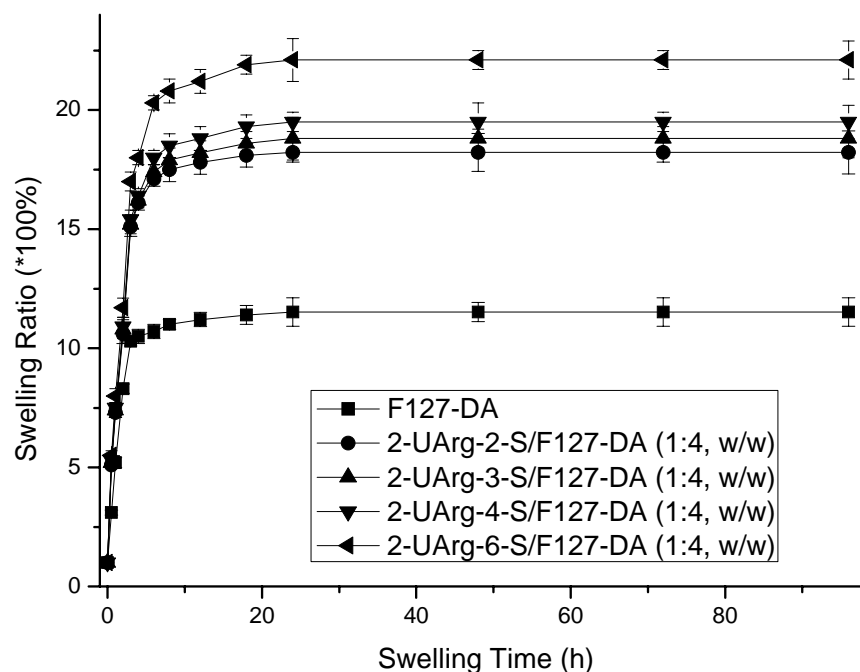


Figure 5.8 Swelling kinetics of Arg-UPEA/Pluronic-DA hydrogels in DI water at room temperature

The equilibrium swelling ratio tests were performed in both deionized water (DI water) and 1X PBS solution. Figure 5.9 showed the equilibrated swelling ratios at room temperature for the hybrid hydrogels with varied compositions. The swelling ratios of Arg-UPEA/Pluronic-DA hybrid hydrogels in DI water were always higher than that of the corresponding hydrogels in PBS solution (Figure 5.9). No significant difference in swelling ratio between PBS and DI media was observed for the pure F127-DA hydrogel (Figure 5.9, a). This observation suggests that the electrolytes in PBS might interfere with the interaction between water molecules and guanidino groups in the hybrid hydrogels. The equilibrated swelling ratios of the hybrid hydrogels generally increase with an increase in the weight feed ratio of Arg-UPEA to

F127-DA either in DI water or PBS solution. For example, in DI water and at room temperature, the hydrogel of 2-UArg-2-S/F127-DA (1/4, w/w) had an equilibrated swelling ratio of 1785 ± 74 %, while the hydrogel of 2-UArg-2-S/F127-DA (2/3, w/w) had an increased equilibrated swelling ratio of 2014 ± 63 %.

We further investigated the effect of molecular structure (y value) of the Arg-UPEA on the swelling property of the hybrid hydrogels at a constant weight ratio of Arg-UPEA to F127-DA. As shown in Table 5.2 and Figure 5.7, the swelling ratio increased as the number of methylene groups in the repeating unit of Arg-UPEA increased. For example, at the feed weight ratio of 1 to 4, in DI water and at room temperature, from 2-UArg-2-S/F127-DA (1/4, w/w) to 2-UArg-6-S/F127-DA (2/3, w/w), the equilibrated swelling ratio increased from 1785 ± 74 % to 2158 ± 98 %. The same trend was observed in PBS buffer, at the feed weight ratio of 1 to 4, from 2-UArg-2-S/F127-DA (1/4, w/w) to 2-UArg-6-S/F127-DA (2/3, w/w), the equilibrated swelling ratio increased from 2407 ± 117 % to 2158 ± 98 %.

The swelling ratio of all Arg-UPEA/Pluronic-DA hybrid hydrogels were higher than that of pure Pluronic-DA hydrogel ($1,144 \pm 17\%$ and $1,150 \pm 24$ % in DI water and PBS, respectively), implying that the incorporation of guanidine group moieties in Arg-UPEA segment did enhance the hydrophilicity and hence swelling of the hybrid hydrogels. This relationship of the effect of precursors' feed ratio and molecular structure on swelling ratio was also found to be consistent with the effect of precursors' feed ratio and molecular structure on the compressive modules of the hydrogels as described later.

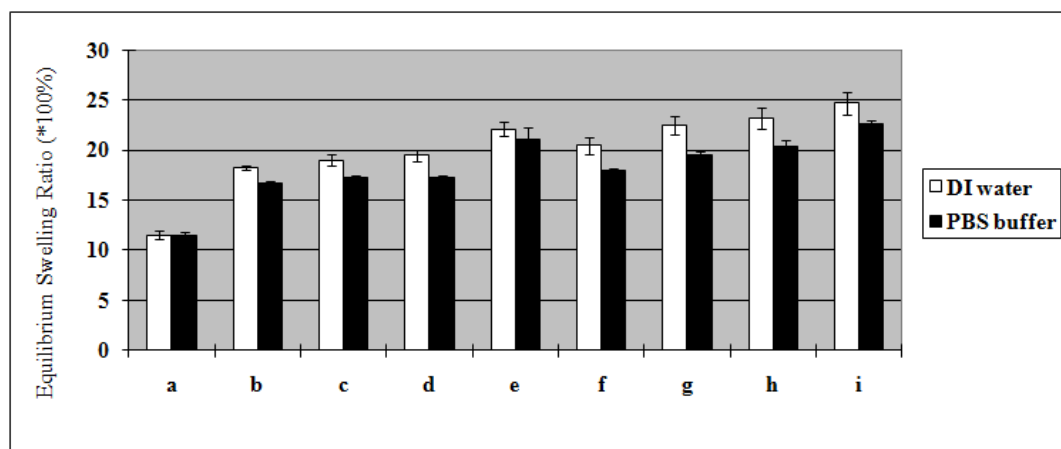


Figure 5.9 Equilibrated swelling ratio of Arg-UPEA/Pluronic-DA hydrogels in PBS (pH=7.4) solution and DI water. a) Pure F127-DA hydrogel (control); b) 2-UArg-2-S/F127-DA (1/4, w/w); c) 2-UArg-3-S/F127-DA (1/4, w/w); d) 2-UArg-4-S/F127-DA (1/4, w/w); e) 2-UArg-6-S/F127-DA (1/4, w/w); f) 2-UArg-2-S/F127-DA (2/3, w/w); g) 2-UArg-3-S/F127-DA (2/3, w/w); h) 2-UArg-4-S/F127-DA (2/3, w/w); i) 2-UArg-6-S/F127-DA (2/3, w/w)

The fast swelling kinetics and high water retention capability of these hybrid hydrogels are due to their hydrophilic characteristic of the hybrid hydrogels. Not all hybrid hydrogels reported in the literature show such fast and high levels of swelling. For example, Guo et al. reported that a relatively hydrophobic hybrid hydrogel family containing phenylalanine based poly (ester amide)/ PEG-DA had much slower swelling kinetics (around 12 hours before the swelling rate leveled off) and lower equilibrium swelling ratios (between 1 and 2,400% in DI water)^{21, 25}.

To further understand the effect of ions on the equilibrium swelling ratio of these hybrid hydrogels, the effect of pH (varied H^+ concentration) on the equilibrium swelling ratios of the Arg-UPEA/Pluronic-DA hybrid hydrogels was examined as shown in Figure 5.10. As expected, the swelling ratios of the hybrid hydrogels were sensitive to the change of pH, and the level of pH sensitivity depended on the feed ratio of Arg-UPEA to Pluronic-DA. In general, all the hybrid hydrogels showed an increase in swelling ratio with a decrease in pH values. The effect of pH on swelling ratio became more pronounced as the Arg-UPEA to Pluronic-DA feed ratio increased because of the increasing of ion density. For example, for the hybrid hydrogels at a feed ratio of 1:4, the equilibrium swelling ratio of 2-UArg-2-S/F127-DA at an acidic pH (pH = 4) was almost 2 times than that at a basic condition (pH = 10). This pH dependent equilibrium swelling ratio of hydrogels was also discussed and reported in other polymeric systems, including PEG-grafted poly (methacrylic acid) (PMAA)³⁴, maleic chitosan hydrogels³⁵, maleic dextran hydrogels³⁶ and poly (vinyl alcohol)-co-maleic anhydride hydrogels³⁷.

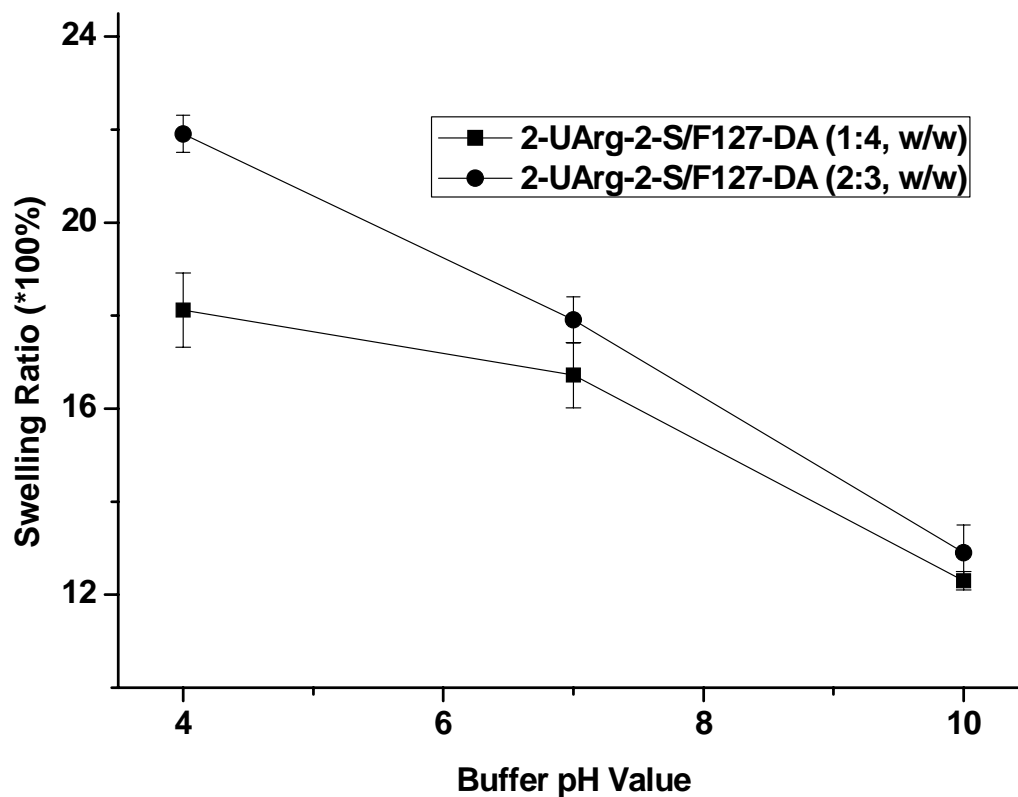


Figure 5.10 the equilibrium swelling ratios of the hybrid hydrogels in aqueous solution as a function of the pH value. a): 2-UArg-2-S/F127-DA (1:4,w/w); b) 2-UArg-2-S/F127-DA (2:3,w/w)

5.D.4 Mechanical Property (Compressive Modulus) of Arg-UPEA/F127-DA Hybrid Hydrogels

For the effect of molecular structure (y value) of Arg-UPEA on the compressive modulus of the hybrid hydrogels, it was found that the compressive modulus decreased with an increase in y value. For example, at a fixed feed weight ratio of Arg-UPEA to F127-DA of 1:4, an increase of y from 2 (2-UArg-2-S/F127-DA), 4 (2-

UArg-4-S/F127-DA) to 6 (2-UArg-6-S/F127-DA), their compressive moduli decreased from 4.85 ± 0.41 , 3.77 ± 0.24 to 2.58 ± 0.25 KPa, respectively. The compressive modulus of Arg-UPEA/F127-DA hydrogel is much less than our previous reported Phe-UPEA/PEG8000-DA system^{21, 22}. For example, for the hybrid hydrogel system of Arg-UPEA/F127-DA with a weight feed ratio of 1:4, the compressive moduli is in the range of 2-5 KPa; while for the Phe-UPEA/PEG8000-DA, the compressive moduli is in the range of 150-900 KPa, which is about 100 times of the Arg-UPEA/F127-DA system. The huge difference is mainly caused by the significant difference between Arg-UPEA and Phe-UPEA. As we know, the mechanical property of hydrogel was very important for the cellular interaction with hydrogel², this Arg-UPEA/Pluronic-DA hybrid hydrogel system offered a variety of mechanical property choices for tissue engineering applications by adjusting the material parameter of the precursor as well as its feed ratio to the co-precursors.

5.D.5 Interior Morphology (SEM) of Arg-UPEA/F127-DA Hydrogels

To further understand the 3-D structure of the Arg-UPEA/F127-DA hybrid hydrogels, the cross-sectional interior morphology of the hybrid hydrogels was examined and shown in Figure 5.11. Compared with a pure F127-DA hydrogel, these Arg-UPEA/F127-DA hybrid hydrogels have larger average pore size and thinner cell wall. For example, a pure F127-DA hydrogel had an average pore size 9 μm , while all Arg-UPEA/F127-DA hybrid hydrogels had the average pore size 11-13 μm . At a fixed feed ratio of precursors, no significant difference of the pore size among the Arg-UPEA/F127-DA hybrid hydrogels due to different y material parameter in the Arg-UPEA precursor. One distinctive morphology of these Arg-UPEA/F127-DA

hybrid hydrogels from a pure F127-DA hydrogel was that the Arg-UPEA/F127-DA hybrid hydrogels showed some nanosize fiber webs entangled with the cells.

5.D.6 Cytotoxicity of Hydrogel Precursors

In this study, the cytotoxicity of hydrogel precursors (Arg-UPEA and Pluronic-DA) was evaluated by MTT assay. As shown in Figure 5.12, under phase-contrast microscopy, no significant morphology change of the fibroblast cell was observed after 48 hrs' treatment with precursors. There were no visible signs of cell rounding or membrane blebbing which would indicate cell death. Results from the MTT assay indicated that more than 95 % cells remain viable when treated with precursor solutions after 48 hrs (Figure 5.13). The minimal cytotoxicity of Arg-UPEAs implies the great potential as a new biomaterial for various biomedical applications.

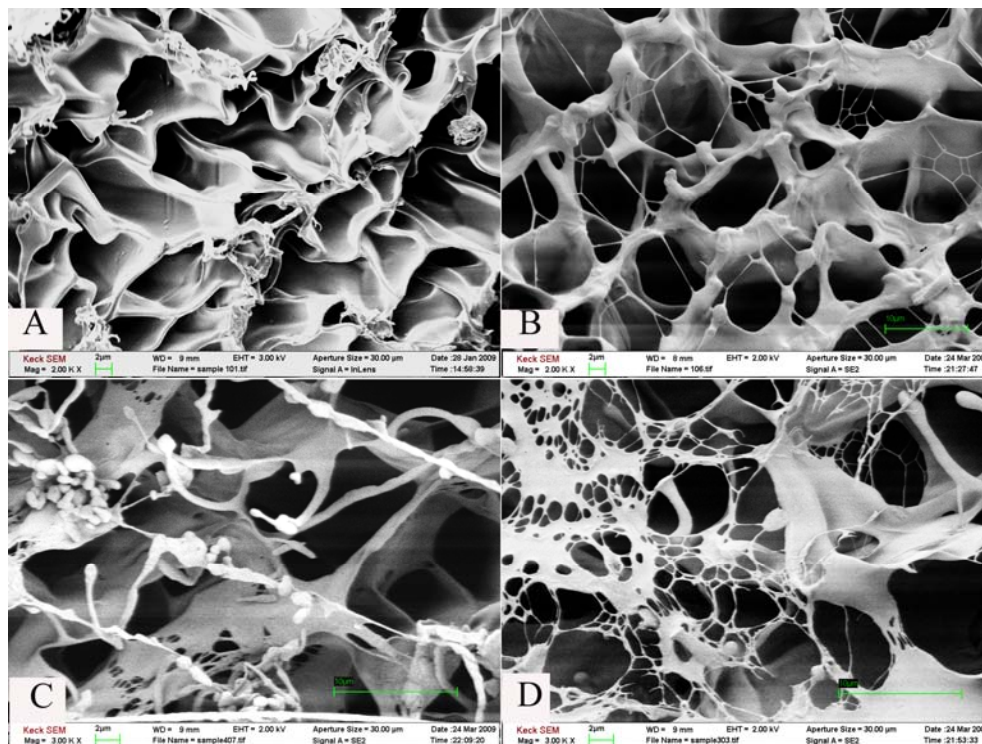


Figure 5.11 SEM images of Arg-UPEA/F127-DA hybrid hydrogels at weight feed ratios of Arg-UPEA/F127-DA of 1/4. A, Pure F127-DA hydrogel; B, 2-UArg-2-S/F127-DA hybrid hydrogel; C, 2-UArg-4-S/F127-DA hybrid hydrogel; D, 2-UArg-6-S/F127-DA hybrid hydrogel. The scale bar (green bar) is 5 μm.

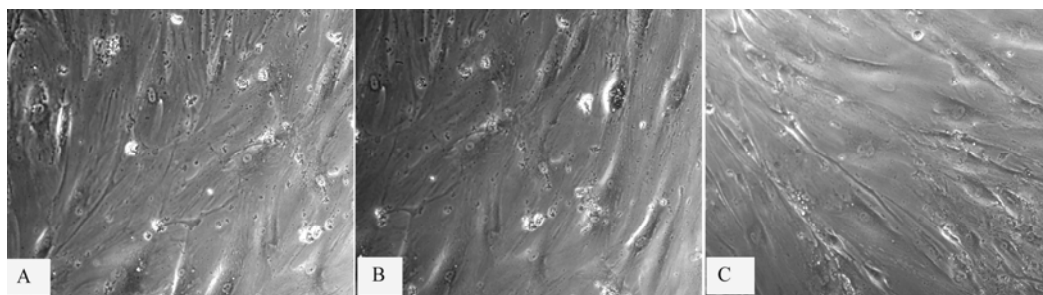


Figure 5.12 Representative micrographs of fibroblast cells after 48 hr culture, 10x. A) cells without any polymer treated (control); B) cells treated with 10 μ L 10 mg/mL F127-DA PBS solution; C) cells treated with 10 μ L 10 mg/mL 2-UArg-2-S PBS solution.

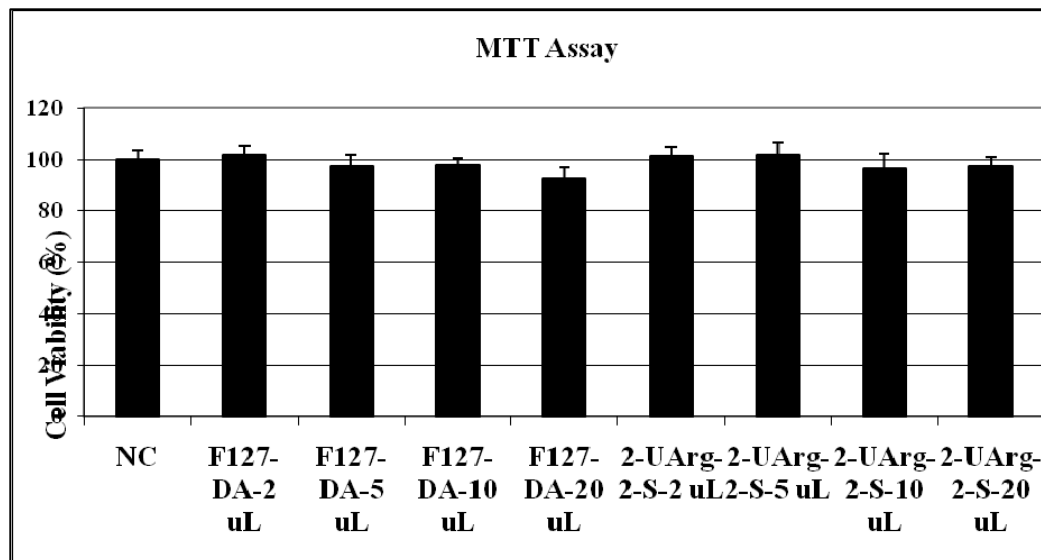


Figure 5.13 Fibroblast cell viability (%) after incubation in various amount of F127-DA and 2-UArg-2-S solution for 48 hours, respectively. NC means negative control: cells without any polymer treatment. Error bars represent mean + SEM, * $P < 0.05$. The number after precursor name means the volume of polymer solution and the concentration for both of precursors is 10 mg/mL.

5.D.7 Human Fibroblast Cell Attachment and Proliferation on Hydrogel Surfaces

To study the cellular interaction with Arg-UPEA/F127-DA hybrid hydrogels, the Detroit 539 human fibroblast cells were cultured on the surface of Arg-UPEA/F127-DA hybrid hydrogels to investigate the cell attachment and proliferation performance. Detroit 539 human fibroblast cells cultured in the 24 well cell culture plate without any other treatment were used as the blank control, and fibroblast cells cultured on the pure F127-DA hydrogel was used as hydrogel control. Figure 5.14 showed one example of the Detroit 539 human fibroblast cells cultured on the surface of 2-UArg-2-S/F127-DA (1/4, w/w) hybrid hydrogel. As shown in Figure 5.14, compared with the pure F127-DA hydrogel control, the hybrid hydrogel had much higher amounts of the attached/proliferated fibroblast cells, and the amounts of the attached/ proliferated fibroblast cells were in the same level as the negative control. These cell morphological data were also confirmed by the MTT assay for the attached/proliferated fibroblast cells (Figure 5.15). Both Figures 5.14 and 5.15 showed that the negative and the hybrid hydrogel have the similar amounts of the attached Detroit 539 human fibroblast cells and both of them had the higher amounts of attached cells than the pure F127-DA hydrogel.

From the aspect of the fibroblast cell morphology, no significant morphology change was detected between the hybrid hydrogel surface (Figure 5.14 C) and the blank control (Figure 5.14 A) after 48 hrs's culture. There were no visible signs of cell rounding or membrane blebbing which would indicate cell death. The Detroit 539 human fibroblast cells attached onto the pure F127-DA hydrogel surface, however, did show some morphology change (Figure 5.14 B). From Figure 5.14 B, we could find that the Detroit 539 human fibroblast cells did not completely attach and spread on the

F127-DA hydrogel surface. Therefore, the introduction of Arg-UPEA to Pluronic-DA hydrogel could enhance the hydrogel's cell attachment and proliferation. For the 2-UArg-2-S/F127-DA hydrogel of a feed ratio of 2 to 3, although the charge density of hydrogel is higher than the 2-UArg-2-S/F127-DA hydrogel with feed ratio of 1 to 4, the cell could not attach and proliferate on the hydrogel surface. The reason could be due to the decreased mechanical property of the hydrogel, further investigation is needed for this phenomenon.

The possible reasons for the observed encouraging Detroit 539 human fibroblast cell attachment and proliferation data from the 2-UArg-2-S/F127-DA hybrid hydrogel system could be due to the excellent biocompatibility and cationic nature of the Arg-UPEA component rather than the F127. Many reported studies have used F127 or F127 derivatives to fabricate hydrogels for tissue engineering applications³⁸⁻⁴⁶. However, those reported studies indicated that F127 hydrogel itself could not support the cell attachment/proliferation well, and many chemical modification methods have been applied to F127 hydrogel system to improve the cellular interaction of F127 hydrogels³⁸⁻⁴⁶. For examples, Lippens et al reported using alanine modified F127 hydrogel for mesenchymal stem cells culture⁴⁶. Vashi et al also reported the stem cells culture on F127 hydrogel surface³⁹. Jung et al reported using TGF- β 1-conjugated biodegradable Pluronic F127 hydrogel for adipose-derived stem cells culture⁴⁴. Park et al reported using chitosan-F127 hydrogels for cartilage regeneration³⁸. Lin et al reported using poly (dimethyl siloxane-urethane)/Pluronic F127 for L929 fibroblast cell culture⁴¹. However, most of the reports indicated that the cell attachment and proliferation performance were not very good on the modified F127 hydrogel surface³⁸⁻⁴⁶. When compared with other reported positively charged hydrogel systems,

such as chemically modified PEGDA and HEMA hydrogel⁴⁷, Arg-UPEA/Pluronic-DA hydrogel system showed much better fibroblast cell attachment performance.

Therefore, this paper reported a different chemical means to modify F127 hydrogel for a significant improvement of BAEC cell attachment and proliferation by the chemical incorporation of cationic Arg-UPEA component. These excellent cell attachment and proliferation performances of Arg-UPEA/F127-DA hybrid hydrogels suggest they may have a great potential as a new type of scaffolds for various biomedical applications. Of course, these cationic Arg-UPEA/Pluronic-DA hybrid hydrogel systems can be further improved with cell-adhesive ligands for additional enhanced cell adhesion.

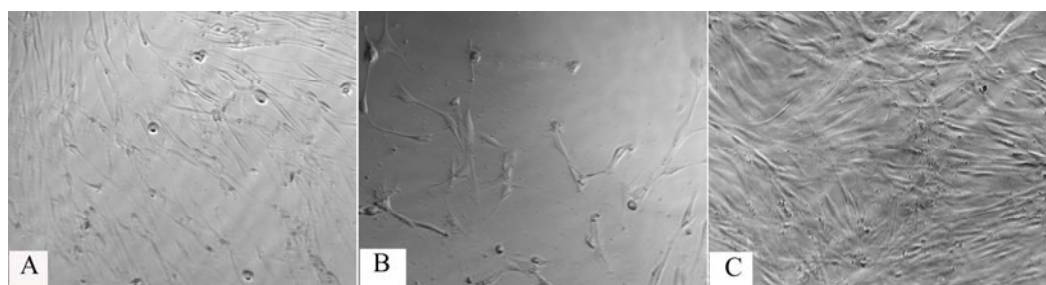


Figure 5.14 Representative micrographs of fibroblast cells after 48 hrs' culture, 10x. A) cells cultured in 24 well cell culture plate without any treatment (control); b) cells cultured on the surface of pure F127-DA hydrogel; c) cells cultured on the surface of 2-UArg-2-S/F127-DA(1/4, w/w) hydrogel.

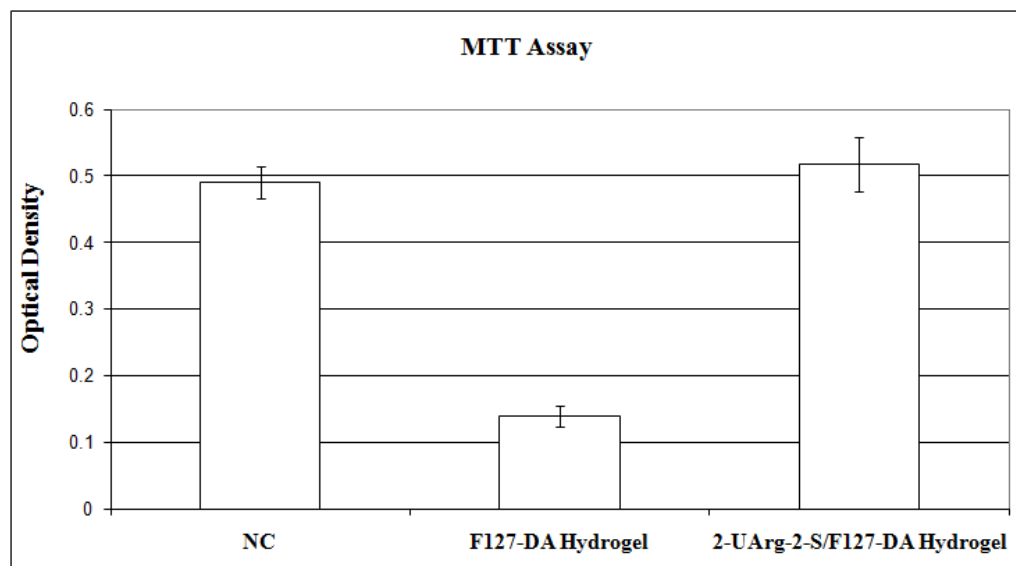


Figure 5.15 MTT Assay for fibroblast cells after 48 hrs' culture on hydrogel surface. 2-UArg-2-S/F127-DA hydrogel had a feed weight ratio of 1 to 4. NC means negative control and the fibroblast cells were cultured directly in the cell culture plate.

5.D.8 BAEC Cell Viability inside Arg-UPEA/F127-DA Hybrid Hydrogels

In order to fully understand the effects of Arg-UPEA on long-term cell behavior, we tested the long term cell viability inside the Arg-UPEA/F127-DA hybrid hydrogel and BAEC cells were encapsulated into the hydrogel and cultured for 2 weeks at 37 °C and 5 % CO₂. The live-dead assay was used to evaluate the BAEC cell viability and was performed according to the manufacturer protocol (LIVE/DEAD® Cell Viability Assay Kit from Invitrogen). As shown in Figure 5.16, after 2 weeks, both inside the pure F127-DA hydrogel and 2-UArg-2-S/F127-DA hybrid hydrogel (1:4 weight feed ratio), almost all of the remaining encapsulated BAEC cells were viable and healthy (stained in green) and no remaining cells were dead or unhealthy (stained in red). The green dots with different sizes mean that the living BAEC cells were in the different sites of the hydrogel. From Figure 5.16, we could found that the hybrid hydrogel had more living cells than the pure F127-DA hydrogel. So the introduction of Arg-UPEA to F127-DA hydrogel could help to increase long term cell viability a lot. And our unpublished data showed that the purity of the precursors, irradiation/crosslinking time and UV power/intensity were very important for the cell viability. High purity and less irradiation time were suggested for the fabrication of cell encapsulated hydrogels. High UV power/intensity normally required less crosslinking time.

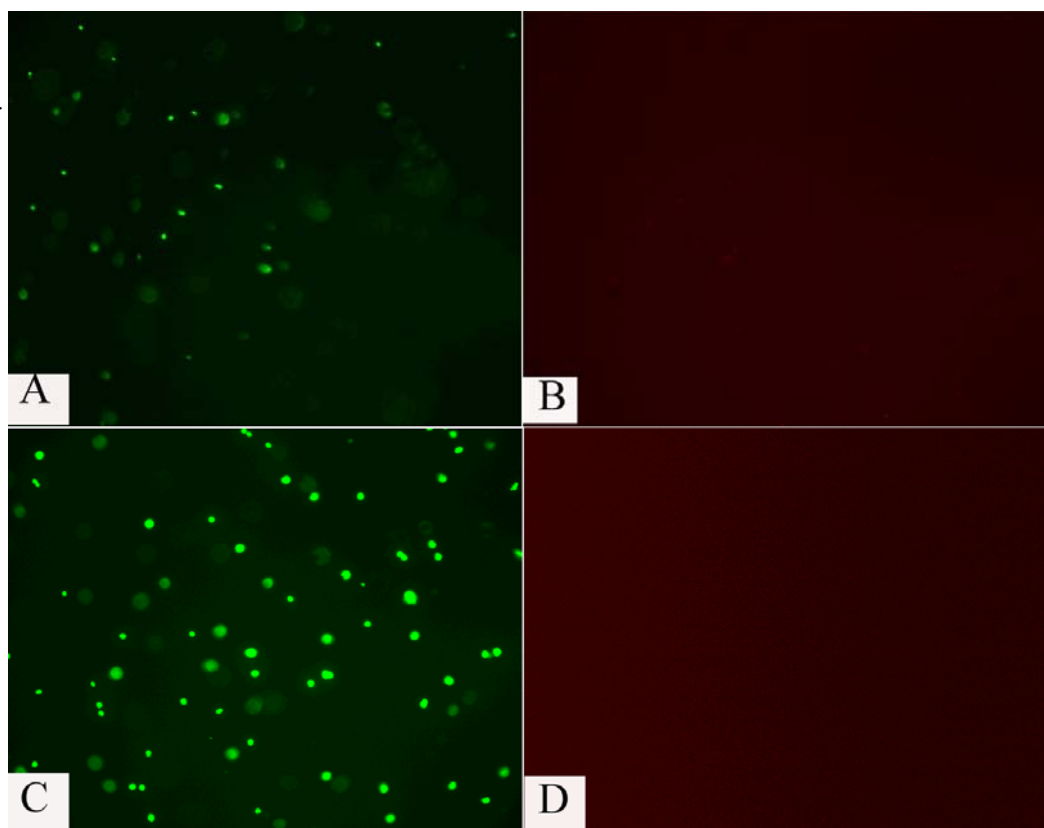


Figure 5.16 live-dead assay for BAEC cells encapsulated in the pure F127-DA hydrogel (A and B) and 2-UArg-2-S/F127-DA hybrid hydrogel (C and D). Green dots in the A and C are for the living cells and red dots in B and D are for the dead cells

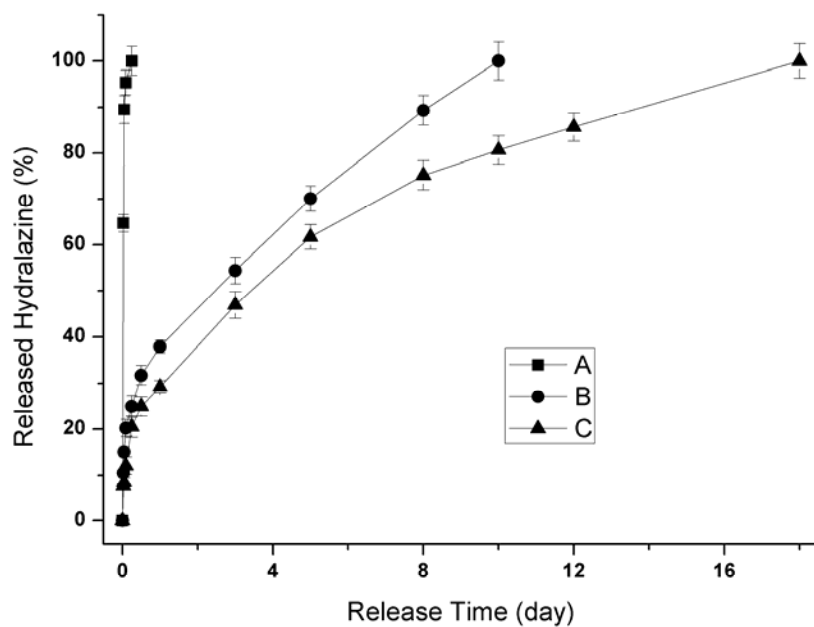


Figure 5.17 Controlled Release of Hydralazine Chlorine Salt via Arg-UPEA/F127-DA Hydrogel: A, hydralazine directly mix with hydrogel precursors; B, hydralazine was preloaded into Hyaluronic acid/Chitosan complex; C, hydralazine was preloaded into Hyaluronic acid/2-UArg-2-S complex

Because of the good biocompatibility, enormous surface area and 3D microporous network structure, hydrogels have been widely used to encapsulate cells to build artificial organs or for other clinical applications^{2, 48-52}. For the fabrication of cell encapsulated hydrogels, many reported studies focused on the collagen or alginate based hydrogels, which were crosslinked by ionic interaction^{2, 48, 49, 53}. In addition to the fact that they can be processed in an aqueous medium, the collagen- or alginate-based hydrogels showed very good cell viability for the encapsulated cells^{2, 48, 49, 53}. Some chemically crosslinked hydrogel systems (such as dextran and hyaluronic based hydrogels) have also been used for encapsulation of fibroblast and macrophage cells and showed good biocompatibility and cell viability^{50, 54}. For example, Weng et al reported using oxidized dextran/hyaluronic acid hybrid hydrogel for the encapsulation of fibroblast cells and macrophage cell lines. After 3 days and one week, the live-dead assay results showed that the encapsulated cells still had pretty high viability^{50, 54} but with some dead cells, and the cell viability was not as good as our UArg-PEA/F127-DA hybrid hydrogel system. The study for a longer cell encapsulation time, such as 2 weeks or longer, was seldom reported as we did in this study. Therefore, the 2 week BAEC cell viability data in the current study offered very useful data for a longer duration of cell encapsulation in the hydrogel system, which is very important for tissue engineering area.

In this study, the newly formulated Arg-UPEA/Pluronic-DA hybrid hydrogel system could offer many choices in terms of water soluble precursors, cationic charge for better cell attachment and proliferation,, mechanical property, hydrophobicity vs. hydrophilicity balance, and pore size.

5.D.9 Controlled Release of Hydralazine (Apresoline) via Arg-UPEA/F127-DA Hybrid Hydrogels

Our preliminary data indicated that the Arg-UPEA/Pluronic-DA hybrid hydrogels had excellent biocompatibility. In order to further expand the applications of Arg-UPEA/Pluronic-DA hybrid hydrogels, we investigated the controlled release of hydralazine (Apresoline) for potential wound healing and other applications. The drug was preloaded into the hydrogel samples (small pellet) by direct mixing and indirect mixing methods. For direct mixing, hydralazine directly mixed with precursors/initiators in distilled water before UV crosslinking. For indirect mixing, hydralazine mixed with cationic polymer (such as chitosan and Arg-PEA/Arg-UPEA) and anionic polymer (such hyaluronic acid and alginate). Then the hydralazine would be loaded into the precipitated polyelectrolyte. Then the polyelectrolyte would mix with precursors/initiators for UV crosslinking. Figure 5.17 showed some release data of hydralazine via Arg-UPEA/F127-DA hydrogels in PBS buffer at 37 °C. The drug content was analyzed by high performance liquid chromatography (HPLC). The elution time of the hydralazine chlorine salt peak is around 2.35-2.37 mins, which was consistent with the previous reports^{55, 56}. The release data from pure F127-DA hydrogel would not be discussed here. From Figure 5.17, it was found that direct mixing hydralazine would lead burst release; all the drugs were released within 4-6 hours. For indirect mixing, around 20-25 % hydralazine would be released within 4-6 hours, which means the burst release was not heavy, and the release time would be within one week or more than two weeks depending on the complex formation. For example, the HA/Chitosan complex showed a slower release trend compared with the Hyaluronic acid/2-UArg-2-S. The reason could be that the chitosan (M_n is around 50 kDa) has higher molecular weight than the 2-UArg-2-S (M_n is around 15 kDa). The

confirmation of the chemical structure of the released hydralazine was carried out by LC-MS method and the major diagnostic MS fragments were analyzed (Table 5.3)^{55, 56}. The result showed that the released hydralazine chlorine salt did not have any change.

Table 5.3 LC-MS Test of Released Hydralazine

m/z	Relative abundance	Structure
161	100%	M ⁺ .

5.E Conclusions

A new family of pH-sensitive and positively charged biocompatible Arg-UPEA/Pluronic-DA hybrid hydrogel was successfully fabricated via UV-photocrosslinking in an aqueous solution with photo-initiator. The physicochemical, swelling, mechanical, and morphological properties were intensively investigated. We demonstrated that by varying the feed ratio of Arg-UPEA to Pluronic-DA and the type of Arg-UPEA, we could finely tune the swelling, mechanical, and morphological properties of a hybrid hydrogel. These results contribute to the understanding of the structure-properties relationship of Arg-UPEA. Biological tests indicated that the introduction of Arg-UPEA to Pluronic-DA hydrogel significantly increase the cell attachment, proliferation and viability. And the controlled release of hydralazine was studied to enlarge the applications of Arg-UPEA/Pluronic-DA hybrid hydrogel, such as the wound healing area.

REFERENCE

1. Peppas, N. A.; Hilt, J. Z.; Khademhosseini, A.; Langer, R., Hydrogels in biology and medicine: from molecular principles to bionanotechnology. *Advanced Materials (Weinheim, Germany)* **2006**, 18, (11), 1345-1360.
2. Lee, K. Y.; Mooney, D. J., Hydrogels for Tissue Engineering. *Chem. Rev. (Washington, D. C.)* **2001**, 101, (7), 1869-1879.
3. Lee, K. Y.; Yuk, S. H., Polymeric protein delivery systems. *Prog. Polym. Sci.* **2007**, 32, (7), 669-697.
4. Varghese, S.; Elisseeff, J. H., Hydrogels for musculoskeletal tissue engineering. *Advances in Polymer Science* **2006**, 203, (Polymers for Regenerative Medicine), 95-144.
5. Estroff, L. A.; Hamilton, A. D., Water Gelation by Small Organic Molecules. *Chem. Rev. (Washington, DC, U. S.)* **2004**, 104, (3), 1201-1217.
6. Yu, L.; Ding, J., Injectable hydrogels as unique biomedical materials. *Chem. Soc. Rev.* **2008**, 37, (8), 1473-1481.
7. Bian, W.; Liao, B.; Badie, N.; Bursac, N., Mesoscopic hydrogel molding to control the 3D geometry of bioartificial muscle tissues. *Nat. Protoc.* **2009**, 4, (10), 1522-1534.
8. Kuang, M.; Wang, D.; Bao, H.; Gao, M.; Moehwald, H.; Jiang, M., Fabrication of multicolor-encoded microspheres by tagging semiconductor nanocrystals to hydrogel spheres. *Advanced Materials (Weinheim, Germany)* **2005**, 17, (3), 267-270.
9. Ruan, C.; Zeng, K.; Varghese, O. K.; Grimes, C. A., A magnetoelastic bioaffinity-based sensor for avidin. *Biosens. Bioelectron.* **2004**, 19, (12), 1695-1701.
10. Lutolf, M. P.; Hubbell, J. A., Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nature biotechnology* **2005**, 23, (1), 47-55.

11. Langer, R., New methods of drug delivery. *Science (Washington, D. C., 1883-)* **1990**, 249, (4976), 1527-33.
12. Nguyen, K. T.; West, J. L., Photopolymerizable hydrogels for tissue engineering applications. *Biomaterials* **2002**, 23, (22), 4307-4314.
13. Choi, S. H.; Lee, S. H.; Park, T. G., Temperature-Sensitive Pluronic/Poly(ethylenimine) Nanocapsules for Thermally Triggered Disruption of Intracellular Endosomal Compartment. *Biomacromolecules* **2006**, 7, (6), 1864-1870.
14. Kim, M. R.; Park, T. G., Temperature-responsive and degradable hyaluronic acid/Pluronic composite hydrogels for controlled release of human growth hormone. *J. Controlled Release* **2002**, 80, (1-3), 69-77.
15. Lee, S.-Y.; Tae, G., Formulation and in vitro characterization of an in situ gelable, photo-polymerizable Pluronic hydrogel suitable for injection. *J. Controlled Release* **2007**, 119, (3), 313-319.
16. Lee Jun, B.; Yoon Jun, J.; Lee Doo, S.; Park Tae, G., Photo-crosslinkable, thermo-sensitive and biodegradable pluronic hydrogels for sustained release of protein. *J Biomater Sci Polym Ed* **2004**, 15, (12), 1571-83.
17. Kabanov, A. V.; Nazarova, I. R.; Astafieva, I. V.; Batrakova, E. V.; Alakhov, V. Y.; Yaroslavov, A. A.; Kabanov, V. A., Micelle Formation and Solubilization of Fluorescent Probes in Poly(oxyethylene-b-oxypropylene-b-oxyethylene) Solutions. *Macromolecules* **1995**, 28, (7), 2303-14.
18. Chu, C.-C., Biodegradable polymeric biomaterials: an updated overview. *Biomaterials* **2007**, 6/1-6/22.
19. Katsarava, R.; Beridze, V.; Arabuli, N.; Kharadze, D.; Chu, C. C.; Won, C. Y., Amino acid-based bioanalogous polymers. synthesis, and study of regular poly(ester amide)s based on bis(alpha -amino acid) alpha ,w-alkylene diesters, and aliphatic

- dicarboxylic acids. *Journal of Polymer Science, Part A: Polymer Chemistry* **1999**, 37, (4), 391-407.
20. Guo, K.; Chu, C. C., Synthesis, characterization, and biodegradation of copolymers of unsaturated and saturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2007**, 45, (9), 1595-1606.
21. Guo, K.; Chu, C. C., Biodegradation of unsaturated poly(ester-amide)s and their hydrogels. *Biomaterials* **2007**, 28, (22), 3284-3294.
22. Guo, K.; Chu, C. C., Controlled release of paclitaxel from biodegradable unsaturated poly(ester amide)s/poly(ethylene glycol) diacrylate hydrogels. *Journal of Biomaterials Science, Polymer Edition* **2007**, 18, (5), 489-504.
23. Guo, K.; Chu, C. C., Copolymers of unsaturated and saturated poly(ether ester amide)s: synthesis, characterization, and biodegradation. *Journal of Applied Polymer Science* **2008**, 110, (3), 1858-1869.
24. Guo, K.; Chu, C. C., Biodegradable and injectable paclitaxel-loaded poly(ester amide)s micropheres: fabrication and characterization. *Journal of Biomedical Materials Research, Part B: Applied Biomaterials* **2009**, 89B, (2), 491-500.
25. Guo, K.; Chu, C. C.; Chkhaidze, E.; Katsarava, R., Synthesis and characterization of novel biodegradable unsaturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2005**, 43, (7), 1463-1477.
26. Huang, S. J.; Bansleben, D. A.; Knox, J. R., Biodegradable polymers: Chymotrypsin degradation of a low molecular weight poly(ester-urea) containing phenylalanine. *Journal of Applied Polymer Science* **1979**, 23, (2), 429-37.
27. Deng, M.; Wu, J.; Reinhart-King, C. A.; Chu, C.-C., Synthesis and Characterization of Biodegradable Poly(ester amide)s with Pendant Amine Functional Groups and in Vitro Cellular Response. *Biomacromolecules* **2009**, 10, (11), 3037-3047.

28. Yamanouchi, D.; Wu, J.; Lazar, A. N.; Craig Kent, K.; Chu, C.-C.; Liu, B., Biodegradable arginine-based poly(ester-amide)s as non-viral gene delivery reagents. *Biomaterials* **2008**, 29, (22), 3269-3277.
29. Pang, X.; Chu, C.-C., Synthesis, characterization and biodegradation of functionalized amino acid-based poly(ester amide)s. *Biomaterials* **2010**, 31, (14), 3745-3754.
30. Pang, X.; Wu, J.; Reinhart-King, C.; Chu, C.-C., Synthesis and characterization of functionalized water soluble cationic poly(ester amide)s. *Journal of Polymer Science, Part A Polymer Chemistry* **2010**, 48, (17), 3758-3766.
31. Tsitlanadze, G.; Kviria, T.; Katsarava, R.; Chu, C. C., In vitro enzymatic biodegradation of amino acid based poly(ester amide)s biomaterials. *Journal of Materials Science: Materials in Medicine* **2004**, 15, (2), 185-190.
32. Tsitlanadze, G.; Machaidze, M.; Kviria, T.; Djavakhishvili, N.; Chu, C. C.; Katsarava, R., Biodegradation of amino-acid-based poly(ester amide)s: In vitro weight loss and preliminary in vivo studies. *Journal of Biomaterials Science, Polymer Edition* **2004**, 15, (1), 1-24.
33. Williams, C. G.; Malik, A. N.; Kim, T. K.; Manson, P. N.; Elisseeff, J. H., Variable cytocompatibility of six cell lines with photoinitiators used for polymerizing hydrogels and cell encapsulation. *Biomaterials* **2004**, 26, (11), 1211-1218.
34. Klier, J.; Scranton, A. B.; Peppas, N. A., Self-associating networks of poly(methacrylic acid-g-ethylene glycol). *Macromolecules* **1990**, 23, (23), 4944-9.
35. Zhong, C.; Wu, J.; Reinhart-King, C. A.; Chu, C. C., Synthesis, characterization and cytotoxicity of photo-crosslinked maleic chitosan-polyethylene glycol diacrylate hybrid hydrogels. *Acta Biomater.* 6, (10), 3908-3918.
36. Kim, S.-H.; Won, C.-Y.; Chu, C.-C., Synthesis and characterization of dextran-maleic acid based hydrogel. *J. Biomed. Mater. Res.* **1999**, 46, (2), 160-170.

37. Liou, F. J.; Wang, Y. J., Preparation and characterization of crosslinked and heat-treated PVA-MA films. *J. Appl. Polym. Sci.* **1996**, 59, (9), 1395-403.
38. Park, K. M.; Lee, S. Y.; Joung, Y. K.; Na, J. S.; Lee, M. C.; Park, K. D., Thermosensitive chitosan-pluronic hydrogel as an injectable cell delivery carrier for cartilage regeneration. *Acta Biomater.* **2009**, 5, (6), 1956-1965.
39. Vashi, A. V.; Keramidas, E.; Abberton, K. M.; Morrison, W. A.; Wilson, J. L.; O'Connor, A. J.; Cooper-White, J. J.; Thompson, E. W., Adipose differentiation of bone marrow-derived mesenchymal stem cells using Pluronic F-127 hydrogel in vitro. *Biomaterials* **2007**, 29, (5), 573-579.
40. Weinand, C.; Pomerantseva, I.; Neville, C. M.; Gupta, R.; Weinberg, E.; Madisch, I.; Shapiro, F.; Abukawa, H.; Troulis, M. J.; Vacanti, J. P., Hydrogel-TCP scaffolds and stem cells for tissue engineering bone. *Bone (San Diego, CA, U. S.)* **2006**, 38, (4), 555-563.
41. Lin, C.-H.; Lin, W.-C.; Yang, M.-C., Fabrication and characterization of ophthalmically compatible hydrogels composed of poly(dimethyl siloxane-urethane)/Pluronic F127. *Colloids Surf., B* **2009**, 71, (1), 36-44.
42. Zheng, X. L.; Wang, X. H.; Gou, M. L.; Zhang, J.; Men, K.; Chen, L. J.; Luo, F.; Zhao, X.; Wei, Y. Q.; Qian, Z. Y., A novel transdermal honokiol formulation based on Pluronic F127 copolymer. *Drug Delivery* **2010**, 17, (3), 138-144.
43. Terada, S.; Yoshimoto, H.; Fuchs, J. R.; Sato, M.; Pomerantseva, I.; Selig, M. K.; Hannouche, D.; Vacanti, J. P., Hydrogel optimization for cultured elastic chondrocytes seeded onto a polyglycolic acid scaffold. *J. Biomed. Mater. Res., Part A* **2005**, 75A, (4), 907-916.
44. Jung, H. H.; Park, K.; Han, D. K., Preparation of TGF-1-conjugated biodegradable pluronic F127 hydrogel and its application with adipose-derived stem cells. *J. Controlled Release* **2010**, 147, (1), 84-91.

45. Park, K.; Jung, H. H.; Son, J. S.; Rhie, J.-W.; Park, K. D.; Ahn, K.-D.; Han, D. K., Thermosensitive and cell-adhesive pluronic hydrogels for human adipose-derived stem cells. *Key Eng. Mater.* **2007**, 342-343, (Advanced Biomaterials VII), 301-304.
46. Lippens, E.; Vertenten, G.; Girones, J.; Declercq, H.; Saunders, J.; Luyten, J.; Duchateau, L.; Schacht, E.; Vlamincx, L.; Gasthuys, F.; Cornelissen, M., Evaluation of Bone Regeneration with an Injectable, In Situ Polymerizable Pluronic F127 Hydrogel Derivative Combined with Autologous Mesenchymal Stem Cells in a Goat Tibia Defect Model. *Tissue Eng., Part A* **2010**, 16, (2), 617-627.
47. Schneider, G. B.; English, A.; Abraham, M.; Zaharias, R.; Stanford, C.; Keller, J., The effect of hydrogel charge density on cell attachment. *Biomaterials* **2004**, 25, (15), 3023-3028.
48. Rouillard, A. D.; Tsui, Y.; Polacheck, W. J.; Lee, J. Y.; Bonassar, L. J.; Kirby, B. J., Control of the Electromechanical Properties of Alginate Hydrogels via Ionic and Covalent Cross-Linking and Microparticle Doping. *Biomacromolecules*, ACS ASAP.
49. Lee, C. S. D.; Gleghorn, J. P.; Choi, N. W.; Cabodi, M.; Stroock, A. D.; Bonassar, L. J., Integration of layered chondrocyte-seeded alginate hydrogel scaffolds. *Biomaterials* **2007**, 28, (19), 2987-2993.
50. Weng, L.; Romanov, A.; Rooney, J.; Chen, W., Non-cytotoxic, in situ gelable hydrogels composed of N-carboxyethyl chitosan and oxidized dextran. *Biomaterials* **2008**, 29, (29), 3905-3913.
51. Wu, J.; Wang, X.; Keum, J. K.; Zhou, H.; Gelfer, M.; Avila-Orta, C.-A.; Pan, H.; Chen, W.; Chiao, S.-M.; Hsiao, B. S.; Chu, B., Water soluble complexes of chitosan-g-MPEG and hyaluronic acid. *Journal of Biomedical Materials Research, Part A* **2007**, 80A, (4), 800-812.
52. Hoffman, A. S., Hydrogels for biomedical applications. *Advanced Drug Delivery Reviews* **2002**, 54, (1), 3-12.

53. Choi, N. W.; Cabodi, M.; Held, B.; Gleghorn, J. P.; Bonassar, L. J.; Stroock, A. D., Microfluidic scaffolds for tissue engineering. *Nat. Mater.* **2007**, 6, (11), 908-915.
54. Weng, L.; Ivanova, N. D.; Zakhaleva, J.; Chen, W., In vitro and in vivo suppression of cellular activity by guanidinoethyl disulfide released from hydrogel microspheres composed of partially oxidized hyaluronan and gelatin. *Biomaterials* **2008**, 29, (31), 4149-4156.
55. Rouan, M. C.; Campestrini, J., Liquid chromatographic determination of dihydralazine and hydralazine in human plasma and its application to pharmacokinetic studies of dihydralazine. *J. Pharm. Sci.* **1985**, 74, (12), 1270-3.
56. Semple, H. A.; Tam, Y. K.; Coutts, R. T., Hydralazine pharmacokinetics and interaction with food: an evaluation of the dog as an animal model. *Pharm. Res.* **1990**, 7, (3), 274-9.

CHAPTER 6

POLY (ESTER AMIDE)-*b*-POLY (ϵ -CAPROLACTONE): SYNTHESIS,
CHARACTERIZATION, FORMULATION, AND IN VITRO CELLULAR
RESPONSE

Jun Wu,¹ Reinhart C. King,¹ C.C. Chu^{1,2}

¹Department of Biomedical Engineering, Cornell University, Ithaca, NY 14853

²Department of Fiber Science and Apparel Design, Cornell University, Ithaca, NY
14853

6.A Abstract

In order to expand the properties and applications of aliphatic polyesters and poly (ester amide)s (PEAs), a new biodegradable block copolymer family, poly (ester amide)-*b*-poly (ϵ -caprolactone) (PEA-*b*-PCL), were synthesized. The resulting copolymers would have both enzymatic biodegradation and hydrolytic degradation properties. These new copolymers were synthesized by first preparing L-Phenylalanine based PEAs (Phe-PEA) with free amine end groups via the solution polycondensation. These amine-terminated PEAs were used to initiate the ring opening polymerization of the ϵ -caprolactone for the synthesis of the PEA-*b*-PCL copolymers. The molecular weight of the PEA-*b*-PCLs block copolymers could be well controlled by adjusting the PEA molecular weight and weight ratio of ϵ -caprolactone and Phe-PEA and ranged from 7 to 50 kg/mol. The new block copolymers' structure and properties were characterized by various physicochemical methods, such as NMR, GPC, and solubility test. To test the polymer's processing capability, PEA-*b*-PCLs were fabricated into different formulations, such as microspheres and electrospun fibers. The in vitro enzymatic biodegradation and some biological studies of PEA-*b*-PCLs were conducted to assess their biological property like supporting for cell attachment and proliferation, and inflammation. The preliminary biological data showed that these new block PEA-PCL copolymers were nontoxic and the bovine aortic endothelial cells (BAEC) showed very good attachment, proliferation and low inflammation response. So the PEA-*b*-PCLs combined the favorable properties of PEA and PCL and expanded the potential applications in biomedical and pharmaceutical areas. The reported synthesis routes of PEA-*b*-PCL could be easily applied to other absorbable aliphatic polyesters to obtain a variety of PEA-*b*-Polyesters.

6.B Introduction

In the past several decades, many absorbable and biocompatible polymers have been developed for biotechnology and pharmaceutical industries¹⁻⁷. Among them, absorbable aliphatic polyesters like polylactide, polyglycolide, poly(ϵ -caprolactone) and their copolymers are the most well-known and widely used because of their good biocompatibility, degradability, consistent mechanical and processing properties¹⁻¹⁵. Although these FDA approved absorbable aliphatic polyesters have been widely used as scaffolds in tissue engineering, drug delivery vehicles and surgical implants, the rapid development of biotechnology needs the new generation polyesters or their derivatives with improved or expanded physicochemical, biological and mechanical property¹⁻¹⁷.

In recent years, many new aliphatic polyester derivatives have been prepared to meet the increasing demands of biomedical field¹. One such new approach is the incorporation of polyether segment into aliphatic polyesters like polyester-*b*-polyether¹⁸⁻²² (e.g., PLA-*b*-PEG) which have been widely investigated in the areas of antibiofouling, self-assembly, drug/gene delivery and nanotechnology^{18, 19}. Another interesting approach is to introduce the natural amino acids into these aliphatic polyester backbones^{1, 6, 15, 23-26}. The incorporation of natural amino acids would bring these aliphatic polyesters many new properties, such as functionality and charge property. One example of this approach is polyester-*b*-poly (amino acid) s, such as PLA-*b*-PLL and PCL-*b*-PLL, which have been widely used as drug delivery vehicles and tissue engineering scaffolds^{15, 23, 24}. However, after such a modification, many of aliphatic polyester derivatives or copolymers lost most of their original properties,

especially the very important mechanical and processing property, which largely limited their applications.

Therefore, a newer approach of integrating absorbable aliphatic polyester with amino acid containing polymers is reported in this study. Instead of using pure poly (amino acid)s to integrate with absorbable aliphatic polyesters, the amino acid-based poly (ester amide) (PEA, Figure 6.1) is used here because of their well-known biological property, biocompatibility and enzymatic biodegradability. Amino acid based PEAs are a family of newly developed biodegradable and biocompatible polymers with ester and amide linkages on the backbones, and have shown very low inflammation response and controllable biodegradability^{6, 27-30}. The PEA backbone consists of nontoxic building blocks like α -amino acids, fatty diols and dicarboxylic acids. The variety of combinations of these 3 building blocks offer many different generations of PEAs for different purposes. The incorporation of PEA into commercially available absorbable aliphatic polyesters could be beneficial to both PEAs and absorbable aliphatic polyesters. To PEAs, the integration with aliphatic polyesters could bring hydrolytic degradation mode in addition to enzymatic biodegradation mode of PEAs. Such integration could also bring stronger mechanical property to PEAs. To aliphatic polyesters, the integration with PEAs could significantly improve the biological property of aliphatic polyesters, such as low inflammation and supporting cell growth. In addition, PEAs could bring useful functional groups like $-\text{COOH}$, $-\text{NH}_2$ to aliphatic polyesters which are well-known for the lack of functional groups³⁰.

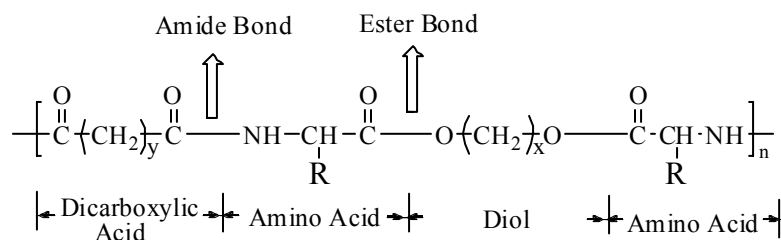


Figure 6.1 Chemical Structure of Poly (ester amide)

In this study, we reported the synthesis, characterization and properties of such a new hybrid block copolymer from aliphatic polyesters and amino acid-based PEAs (PEA-*b*-PCL). The resulting new hybrids could be fabricated into microspheres and electrospun fibrous membranes. These PEA-*b*-PCLs were also tested by many biological assays to determine their cellular responses, such as enzyme biodegradation, cell attachment, cell proliferation and *in vitro* inflammation assays. The biological data obtained suggest that these new hybrid copolymers were nontoxic to cells and the introduction of PEA into PCL could promote the cell attachment and proliferation, and significantly reduce the inflammation response of PCL.

6.C Experimental

6.C.1 Materials

L-Phenylalanine (L-Phe), *p*-toluenesulfonic acid monohydrate, adipoyl chloride, sebacoyl chloride, 1, 4-butanediol, 1, 6-hexanediol and *p*-nitrophenol were all purchased from Alfa Aesar (Ward Hill, MA) and used without further purification. ϵ -caprolactone, Tin(II) 2-ethylhexanoate (Sn(Oct)₂), Poly (ϵ -caprolactone) (PCL, M_n =80,000) were purchased from Aldrich and used directly. Poly (n-butyl methacrylate) (PBMA) was purchased from Polysciences and used directly. Triethylamine from

Fisher Scientific (Fairlawn, NJ) was dried by refluxing with calcium hydride, and then distilled before use. Solvents like toluene, ethyl acetate, acetone; 2-propanol, dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from VWR Scientific (West Chester, PA) and were purified by standard methods before use. Other chemicals and reagents if not otherwise specified were purchased from Sigma (St. Louis, MO).

α -Chymotrypsin (Type II, from bovine pancreas, 66 units/mg, solid) was purchased from Sigma Chemical Co. (St. Louis, MO) and chosen as the model enzyme because it could hydrolyze ester linkages at C-terminal of hydrophobic α -amino acids like L-phenylalanine. PBS buffer (0.1M, pH 7.4) was used for the biodegradation study of PEA-*b*-PCL block copolymers.

6.C.2 Synthesis of monomers and polymers

The general scheme of the synthesis of PEA-*b*-PCL was divided into the following two major tasks: 1) the synthesis of poly (ester amide)s with free amine end groups via a solution polycondensation; 2) the synthesis of PEA-*b*-PCL block copolymers through the ring opening polymerization of ϵ -caprolactone via the initiation by the free amine end groups of the PEA. For the synthesis of PEA, two types of monomers were synthesized and then polycondensed in a solvent. The two monomers were: di-*p*-nitrophenyl ester of dicarboxylic acids (**I**) (Figure 6.2), and tetra-*p*-toluenesulfonic acid salts of bis (L-phenylalanine), α , ω -alkylene diesters (**II**) (Figure 6.3). These 2 monomers, **I** and **II** were then polycondensed into low molecular weight PEAs having free amine end groups (**III**) (Figure 6.4). The details of the PEA monomers and polymer synthesis could be found in our prior publications²⁷. The main difference

from the prior published procedures is the reaction time. In this study, the reaction time was shortened so that low molecular weight PEAs having free amine end groups could be obtained.

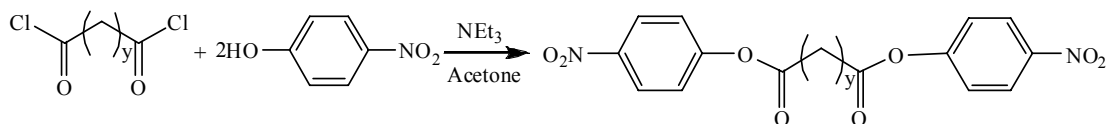


Figure 6.2 Synthesis of Monomer **I**: Di-p-nitrophenyl Ester of Dicarboxylic Acids

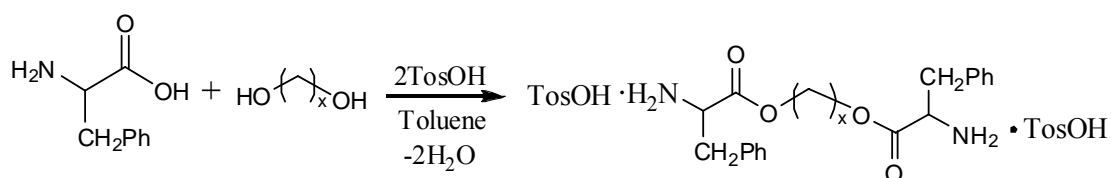


Figure 6.3 Synthesis of Monomer **II**: Di-p-toluenesulfonic Acid salt of Bis (L-Phenylalanine) Alkylene Diesters

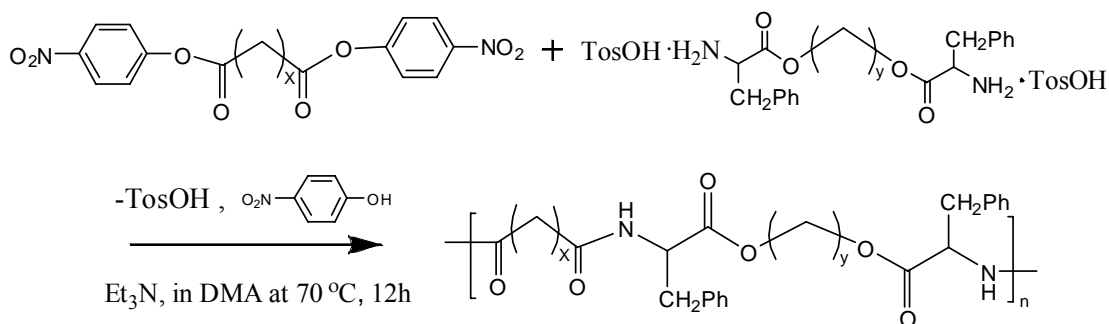


Figure 6.4 Synthesis of PEAs via solution polycondensation of monomers **I** and **II**.

6.C.2.a Synthesis of Monomers: Di-*p*-nitrophenyl Ester of Dicarboxylic Acids (I) and Di-*p*-toluenesulfonic Acid salt of Bis (L-Phenylalanine) Alkylene Diesters (II)

Di-*p*-nitrophenyl esters of dicarboxylic acids were prepared by reacting dicarboxylic acyl chloride varying in methylene length with *p*-nitrophenol as previously reported^{6, 27-32}. Two monomers **I** were made in this study: di-*p*-Nitrophenyl Adipate (**NA**), $x=4$; and di-*p*-Nitrophenyl Sebacate (**NS**), $x=8$ (x indicates the numbers of methylene group in the diacid). The preparation of di-*p*-toluenesulfonic acid salt of bis (L-phenylalanine) alkylene diesters was also followed the previous publications^{6, 27-32}. Two monomers **II** were made in this study: tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) butane diesters, **Phe-4**, $x=4$, and tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) hexane diesters, **Phe-6**, $x=6$.

6.C.2.b Synthesis of PEA (III) by Solution Condensation of (I) and (II)

PEAs were prepared by the solution polycondensation of the above monomers **I** and **II** (**Phe-4**, **Phe-8** and **NA**, **NS**) at different combinations and molar ratios. The PEAs synthesized are summarized in Table 6.1 and are labeled as x -Phe- y , where x and y are the number of methylene group in diacid and diol, respectively. In this report, each type of PEA was made at two different molecular weights, one at 4-5,000; and another at 7-9,000. An example of the synthesis of 8-Phe-4 of number average molecular weight (M_n) around 4,000 via solution polycondensation was given here. Monomers **NS** (0.8 mmol) and **Phe-8** (1.0 mmol) in 1.5 mL of dry DMSO were mixed well and the mixture solution was then heated up to 75 °C under magnetic bar stirring to obtain a uniformed mixture. Triethylamine (0.31 mL, 2.2 mmol) was added drop by drop to the mixture at 75 °C with vigorous stirring until the complete dissolution of

the monomers. The solution became viscous and the color turned into yellow within several minutes. The reaction vial was then kept for 12 hrs at 75 °C in a thermostat oven without stirring. The resulting PEA polymer was precipitated from the reaction solution by adding 300 mL cold ethyl acetate, and the product was purified by Soxhlet extractor using ethyl acetate as solvent for 24 h. The final dried Phe-PEA products are yellow or pale yellow solid and dried *in vacuo* at room temperature.

Table 6.1 Phe-PEAs prepared by different combination of monomers

	y=4	y=6
x=4	4-Phe-4	4-Phe-6
x=8	8-Phe-4	8-Phe-6

6.C.3 Synthesis of *Phe-PEA-b-PCL*

The PEA-*b*-PCL block copolymers were synthesized by the ring-opening polymerization of ϵ -caprolactone (ϵ -CL) using the free NH₂ end groups of Phe-PEA as the macro-initiator with Sn (Oct)₂ as the catalyst (Figure 6.5). In this report, all PEAs used for preparing PEA-*b*-PCL were phenylalanine based PEAs. The polymerization was carried out in a 100 mL 3-neck round bottom flask under dry nitrogen atmosphere at 130 °C.

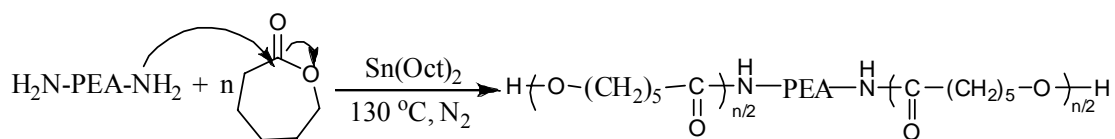


Figure 6.5 Synthesis of PEA-*b*-PCL from the ring-opening polymerization of ϵ -caprolactone via the macro-initiator, H₂N-PEA-NH₂

An example of the synthesis of 8-Phe-4-*b*-PCL from 8-Phe-4 of M_n 4,000, and ϵ -caprolactone at the 8-Phe-4 to ϵ -CL feed weight ratio of 1 to 6 is given here. 8-Phe-4 (2.00 g) and ϵ -CL (12.00 g) were added into a 100 mL 3-neck round bottom flask under dry nitrogen atmosphere. The catalyst, Sn (Oct)₂, was dissolved in dried THF to make a 10 wt% solution and was added to the reaction mixture with a weight ratio of 1:500 [Sn (Oct)₂ to ϵ -CL]. Then, the temperature of the flask was increased to 130 °C under slow magnetic bar stirring and the duration time for the polymerization is 16 h. After that, the final solid product was dissolved in chloroform and precipitated in cold ethyl ether, purified twice, and the final product (white-yellow solid powder) was dried under vacuum at room temperature to constant weight. The yield is around 90-95 %.

6.C.4 Characterization

The physicochemical properties of the prepared monomers and polymers were characterized by various standard methods. For Fourier transform infrared (FTIR) characterization, the samples were ground into powders and mixed with KBr at a sample/KBr ratio of 1:10 (w/w). FTIR spectra were then obtained with a Perkin-Elmer (Madison, WI) Nicolet Magana 560 FTIR spectrometer with Omnic software for data

acquisition and analysis. NMR spectra were recorded with a Varian (Palo Alto, CA) Unity Inova 400-MHz spectrometer operating at 400 for ^1H NMR. Deuterated chloroform (CHCl_3-d ; Cambridge Isotope Laboratories, Andover, MA) with tetramethylsilane as an internal standard or deuterated dimethyl sulfoxide ($\text{DMSO}-d_6$; Cambridge Isotope Laboratories) was used as the solvent. MestReNova software was used for the data analysis. The solubility of the polymers in common organic solvents at room temperature was assessed by using 10.0 mg/mL as a solubility standard to determine whether a polymer was soluble or not in a solvent. The thermal properties of the synthesized PEA-*b*-PCLs were characterized with a DSC 2920 (TA Instruments, New Castle, DE). The measurements were carried out from -30 to 200 °C at a scanning rate of 10 °C/min and at a nitrogen gas flow rate of 25 mL/min. TA Universal Analysis software was used for thermal data analysis.

The number-average molecular weight (M_n), weight-average molecular weight (M_w), and polydispersity (MWD) of the synthesized PEAs and PEA-*b*-PCLs were determined with a model 510 gel permeation chromatograph (GPC, Waters Associates, Inc., Milford, United States) equipped with a high-pressure liquid chromatography pump, a Waters 486 UV detector, and a Waters 2410 differential refractive index detector. Tetrahydrofuran (THF) was used as the eluent (1.0 mL/min). The columns were calibrated with polystyrene standards with a narrow MWD.

The static contact angle of the polymers was measured by a Ramé-Hart Model 500 Advanced Goniometer/Tensiometer. The round micro cover glasses (diameter, 12 mm, no.2,VWR, West Chester, PA) were coated with the polymer in DMF solutions (2 wt%) and vacuum drying before testing. The static contact angle was measured by dropping the distilled water (4 μL) on the polymer coated surface. Each polymer

coating was measured for triple times.

6.C.5 Formulation of PEA-*b*-PCL into Microspheres

PEA-*b*-PCL microspheres were formulated by an oil-in-water (O/W) emulsion/solvent evaporation technique. For the PEA-*b*-PCL used here, the PEA was from 8-Phe-4 with M_n of 4,000, and the feed weight ratio of 8-Phe-4/ ϵ -CL = 1:6). A predetermined amount of PEA-*b*-PCL polymer was first dissolved in 5.0 mL dichloromethane (DCM) or chloroform. The resultant polymer solution was then poured rapidly into 100.0 mL of aqueous PVA solution with a predetermined concentration. The O/W emulsion was achieved by a thorough stirring with a homogenizer (PowerGen Model 35, Fisher Scientific) at 10,000 or 20,000 rpm for 5-10 min. The emulsified system was then stirred with a magnetic stirrer for the evaporation of the organic solvent (DCM or chloroform) for 3 h, the dispersed microdroplets finally solidified in the aqueous PVA solution. The microspheres were washed by the distilled water three times to remove residual PVA and then collected by centrifugation (13,000 rpm). After the microspheres were freeze-dried in a Labconco FreeZone Benchtop Freeze Dry System (Kansas City, MO) under vacuum at -48 °C for 72 h, they were stored in a refrigerator at 4 °C for the future characterizations and tests.

6.C.6 Formulation of Phe-PEA-*b*-PCL into Microfibers by Electrospun Method

A mixed solvent of chloroform and DMF with a weight ratio of 4.0 (chloroform/DMF) was used to dissolve the PEA-*b*-PCL to form a 20 wt% polymer solution. For the PEA-*b*-PCL used here, the PEA was from 8-Phe-4 with M_n of 4,000, and the feed

weight ratio of 8-Phe-4- ϵ -CL = 1:6). The polymer solution was delivered by a programmable pump (Harvard Apparatus, MA) to the exit hole of the electrode (needle with a hole having a diameter of 0.7 mm). The flow rate was set at 50.0 μ L/min. A positive high-voltage supply (Glassman High Voltage Inc.) was used to supply the voltage in a range of 15-20 kV. The fibers were collected on a collection plate. The distance of electric field (from the electrode to collector) was fixed at 120 mm. Scanning electron microscopy (SEM) was used to examine the surface morphology of PEA-*b*-PCL microspheres and electrospun fibers. The dried microsphere or fiber samples were fixed on aluminum stubs and coated with gold under vacuum for 30 s for SEM observation (Leica S440, Germany).

6.C.7 In Vitro Enzymatic Biodegradation of PEA-b-PCL

Polymer film samples (around 200 mg, round shape and same thickness) was added into a small vial containing 10 mL of PBS buffer (pH= 7.4, 0.1 M) with T. The mixture was then incubated at 37 °C with a constant reciprocal shaking (ca. 100 rpm). At the end of predetermined period, the polymer film samples were removed by filtration, then washed with distilled water for 3 times, and dried in vacuum at 35 °C for 24 h to completely remove the residue water. The immersion media were refreshed at every test time and every 48 h in order to maintain the enzymatic activity. The degree of biodegradation was estimated from the weight loss of the polymer based on the following equation:

$$W_t (\%) = (W_o - W_t) / W_o \times 100$$

Where W_o is the original weight of the dry polymer sample before immersion, and W_t is the dry polymer sample weight after incubation for t hours/days (with or without enzyme). The averaged weight loss of three specimens was measured for each sample.

6.C.8 Cell Culture

The interaction of PEA-*b*-PCLs with cells was preliminarily studied to determine the level of cell attachment, proliferation and inflammation. Bovine Aorta Endothelial Cells (BAEC) was used as the model cells for attachment and proliferation tests. BAEC were purchase from VEC Technologies and maintained at 37 °C in 5 % CO₂ in Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10 % Fetal Clone III (HyClone, Logan, UT), and 1 % each of penicillin–streptomycin, MEM amino acids (Invitrogen, Carlsbad, CA), and MEM vitamins (Mediatech, Manassas, VA). BAECs were used from passages 8–12. J774 mouse peritoneal macrophages were used as the model cells for *in vitro* inflammation response tests, which were obtained from ATCC and cultured at 37 °C in 5 % CO₂ in DMEM supplemented with 10 % FBS. J774s were used from passage 5-10. For all the cells, the cell media was changed every 2 days. Cells were grown to 70 % confluence before splitting or harvesting.

6.C.9 Cell Attachment and Proliferation on PEA-*b*-PCL

The evaluation of the endothelial cell attachment and proliferation capability on the PEA-*b*-PCL surface was performed by cell proliferation assay with subsequent MTT assay. The round micro cover glasses (diameter, 12 mm, no.2,VWR, West Chester, PA) were coated with polymer DMF solution (2 wt%) and vacuum drying. The following polymers were tested: PCL, PEA-*b*-PCL, mixtures of PEA and PCL. Commercial available PBMA was selected as the control. After drying, the polymer coated glass coverslips were placed onto the bottom of the 24-well cell culture plates and were sterilized for overnight under a UV irradiation before use.

Cells at an appropriate cell density concentration (20,000 cells/well) were seeded onto each test well in 24-well plates (BD Falcon™, polystyrene treated) and then incubated in a 37 °C, 5 % CO₂ incubator. Cell media was changed every day. After the predetermined periods (48 h and 96 h), the cell culture plates were removed from the incubator. Cell morphology was recorded under an optical microscope. The media from the wells were then aspirated, and 0.5 mL fresh media were added to each well. After that, 40 µL of MTT solution (5 mg/mL) was subsequently added to each well, followed by 4 hr incubation at 37 °C, 5 % CO₂. The cell culture medium was carefully removed and 400 µL of acidic isopropyl alcohol (with 0.1 M HCl) was added to dissolve the formed formazan crystals. The plate was slightly shaken for 30 mins and 100 µL solution was transferred from each well to a 96 well cell culture plate. Optical density (OD) of each well was measured at 570 nm (subtract background reading at 690 nm) by using a microplate reader.

6.C.10 In Vitro Measurement of Inflammatory Response of PEA-b-PCL

J774 macrophages were seeded at 10,000 cells/well onto 12 mm polymer-coated glass coverslips in 24-well tissue culture plates. A plain glass coverslip was used as a negative control. Positive controls were glass coverslips in media containing Lipopolysaccharide (LPS, from E. coli 0111:B4, Sigma-Aldrich, St. Louis, MO) at final concentrations of 1.25 µg/mL and 5 µg/mL³³. A plain glass coverslip in media alone was used as a cell-free negative control. PEA-*b*-PCL (PEA was from 8-Phe-4 with M_n of 4,000, and the feed weight ratio of 8-Phe-4/ ε-CL = 1:6) was selected for this test. PCL and PBMA were used as polymer control. Macrophage activation after 48 hours incubation was measured using an ELISA kit to measure mouse TNF-α.

release (Invitrogen, Carlsbad, CA) according to the manufacturer's suggested protocol and $N = 3$. TNF- α concentrations were calculated from a standard curve using a 4-parameter standard curve-fitting algorithm (Gen5 software, BioTek Instruments, Winooski, VT). All samples and controls were read in duplicate on a 96-well plate reader at 450 nm and referenced against a chromogen blank.

6.C.11 Statistics

Where appropriate, the data are presented as mean \pm standard error of the mean calculated over at least three data points. Significant differences compared to control groups were evaluated by unpaired Student's t-test or Dunnet test at $p < 0.05$, and between more than two groups by Tukey's test with or without one-way ANOVA analysis of variance. JMP software (version 8.0, from SAS Company) was used for data analysis.

6.D Results and Discussion

6.D.1 Synthesis and Characterization of PEAs with Functional End Groups

In this study, four types of monomers (two monomers **I**, NA, NS; and two monomers **II**, Phe-4 and Phe-6) were synthesized according to our previously published procedures for preparing PEA with free amine end groups²⁷. All these monomers were prepared with high yields and easily purified by re-crystallization. The chemical structure and purity had been confirmed by ¹HNMR, FTIR and DSC. All the data were consistent with the published data^{27, 29, 34}.

Unlike the previous reported Phe-PEAs, which had higher molecular weight (M_n 25-30 kg/mol measured by GPC in THF) and less active end groups, in this study, Phe-PEAs with active end amine groups and controlled molecular weight (MW) were required as a macro-initiator to successfully prepare the PEA-*b*-PCL via ring-opening polymerization.

H_2N -PEA- NH_2 was prepared according to the reaction scheme in Figure 6.4. Based on the Carothers Equation [$X_n = (1+r)/(1+r-2rp)$], the molecular weight and end functional groups of polymers made from polycondensation method could be affected or controlled by the following parameters: the molar ratio between 2 monomers (r = monomer **II**/monomer **I** in this study), reaction temperature (T) and time (t), catalyst type and its concentration, monomer concentration, etc. For example, according to the Carothers Equation, if the molar ratio r was changed, the MW of the prepared PEA would be affected and the end groups could be controlled. For examples, if $r=1.0$, the molecular weight will be maximum and the end groups would be one free amine group and one acid group; if $r>1.0$, the molecular weight will decrease with the increasing of r value, and the end groups would be 2 free acid groups; if $r<1.0$, the molecular weight will decrease with the decreasing of r value, and the end groups would be 2 free amine groups. The details of the relationship between r and end groups are summarized in Table 6.2. All these reaction parameters were intensively studied, and it was found that r and t were the key factors affecting the PEA MW and end functional groups. In this study, we only focused on r , the other parameters were fixed at the same values as the previous study²⁷. After optimization, the reaction conditions are: reaction temperature: 70 °C; concentration of each monomer: 1.0-1.5 mmol/mL; the reaction medium: DMA; catalyst (acid acceptor): NEt_3 , reaction time: 6 h. For r , after optimization, it was found that the MW and end functional groups have

the following relationships with r as shown in Table 6.3. In Table 6.3, the Phe-PEAs used to make PEA-*b*-PCL are 8-Phe-4 with r value equals to 0.8 and 0.9.

In this study, all the prepared Phe-based PEAs have 2 free NH₂ end groups and hence the subsequent PEA-*b*-PCL synthesized are A-B-A type (A: PCL; B, PEA) block copolymer. All the PEAs are prepared with high yields (> 80 %) under the optimized reaction conditions. For the chemical structure identification of the prepared Phe-PEAs, the structure was confirmed by DSC, ¹H-NMR and FTIR spectra. The ¹H-NMR and FTIR spectra were consistent with the previous reports²⁷.

6.D.2 Synthesis and Characterization of PEA-b-PCL

As shown in Figure 6.5, the PEA-*b*-PCLs were prepared by ring opening polymerization of ϵ -caprolactone with H₂N-PEA-NH₂ as the macro-initiator and Sn(Oct)₂ as catalyst. The polymerization conditions were optimized and it was found that the following conditions were good for the PEA-*b*-PCLs copolymer synthesis: reaction temperature: 130 °C; polymerization duration time: 16 h; molar ratio of catalyst to monomer=1:500; N₂ protection. The chemical structure and molecular weight of the new copolymers were characterized and confirmed by ¹H-NMR (Figure 6.6) and GPC (Table 6.4). Figure 6.6 showed an example of the ¹H-NMR spectrum of PEA-*b*-PCL (8-Phe-4-*b*-PCL synthesized from 8-Phe-4 of M_n 4.1k and PEA/ ϵ -CL=1:1, w/w); all the ¹H-NMR peaks of PEA-*b*-PCL were identified, and the integration area ratios were consistent with the calculated theoretical ratios. The ¹H-NMR peaks marked with numbers from 1 to 11 are assigned to the corresponding protons of 8-Phe-4-*b*-PCL as shown in Figure 6.6.

For the GPC data, the GPC traces are unimodal with no signal of coexisting low or high molecular weight species that may be produced from uncontrolled polycondensation. It was found that a successfully prepared PEA-*b*-PCL copolymer only showed one main GPC peak; if the polymerization was failed or did not complete, the GPC would show two or more peaks. For the two peak case, one peak was from PCL and the other one was PCL-*b*-PEA, which were observed when the weight ratio of ϵ -CL to H₂N-PEA-NH₂ (8-Phe-4) was too large (more than 6) or the M_n of PEA was too big.

The key factors for the successful PEA-*b*-PCL synthesis are the MW and end functional NH₂ groups of the H₂N-PEA-NH₂. It was found that if the M_n of H₂N-PEA-NH₂ was greater than 15 kg/mol, the side reaction would happen during the synthesis of PEA-*b*-PCL and significant amounts of byproducts existed in the final product, which could not be separated from the PEA-*b*-PCL. The majority of the byproduct was PCL prepared from the polymerization of ϵ -CL monomers without PEA initiation. All these evidences have strongly supported the anticipated molecular structure of PEA-*b*-PCL.

Table 6.2 Relationship between *r* and end groups of Phe-PEAs

<i>r</i> value	End groups of Phe-PEAs
=1.0	One NH ₂ group and one COOH group
>1.0	Two COOH groups
<1.0	Two NH ₂ groups

Table 6.3 Information of some prepared Phe-PEAs

PEA	r	Reaction Time(h)	M _n (kg/mol)	End Functional Groups	Yield (%)
4-Phe-4	0.8	8h	3.5-4.5	2 NH ₂ groups	85
4-Phe-4	0.9	8h	7-8	2 NH ₂ groups	87
4-Phe-6	0.8	8h	3.5-4.5	2 NH ₂ groups	93
4-Phe-6	0.9	8h	7-8	2 NH ₂ groups	84
8-Phe-4	0.8	8h	3.5-5	2 NH ₂ groups	91
8-Phe-4	0.9	8h	7-9	2 NH ₂ groups	89
8-Phe-6	0.8	8h	3.5-5	2 NH ₂ groups	85
8-Phe-6	0.9	8h	7-9	2 NH ₂ groups	83

Many types of PEA-*b*-PCL were prepared and some examples were given in Table 6.4. The data in Table 6.4 indicate that an increase in the feed weight ratio (WR) of ϵ -CL/ H₂N-PEA-NH₂ in the ring polymerization would lead to an increase in the M_n of the PEA-*b*-PCL obtained and was consistent with the calculated theoretical M_n (from the M_n of PEA (8-Phe-4) and feed weight ratio of 8-Phe-4 to ϵ -CL). When the feed WR reached certain values (such as 1:9), the M_n of PEA-*b*-PCL would also reach a peak value and would not increase with the increasing of WR. The peak M_n values of PEA-*b*-PCL were found to be affected by the M_n of H₂N-PEA-NH₂ (8-Phe-4). For example, for 8-Phe-4 with M_n of 7-9,000, it's around 50 kg/mol. The type of H₂N-PEA-NH₂, however, did not show any obvious effect on such a molecular weight relationship. For this phenomenon, it's reasonable that the PEA-*b*-PCL 's MW has some limitations because if the WR of ϵ -CL / H₂N-PEA-NH₂ (8-Phe-4) is too high, the polymerization will need longer time; and density of the active NH₂ is not high enough, then ϵ -CL could be polymerized without the initiation from the NH₂ of PEA. All the MWD of PEA-*b*-PCL is around the range of 1.30-1.60, which is similar to the MWD of Phe-PEA²⁷.

6.D.3 Solubility

The solubility of PEA-*b*-PCL can greatly affect their potential biomedical applications. All the PEA-*b*-PCL polymers synthesized in this study were insoluble in non- polar or weak polar solvent like ether and ethanol; but soluble in polar organic solvent like chloroform, DMF and THF (Table 6.5). The PEA-*b*-PCL copolymers did not show significant solubility difference from either the PEA or PCL. Among all the PEA-*b*-PCL copolymers, they did not show significant difference in the solubility property, even though these copolymers had different types of PEAs and feed weight

ratio to PCL. This is because the solubility difference between PCL and x-Phe-y PEA is not big. The PEA-*b*-PCLs showed similar solubility as the pure PCL since the majority part of the copolymer is PCL. When compared with 8-Phe-4, 8-Phe-4-*b*-PCLs dissolved in acetone; while 8-Phe-4 could not.

6.D.4 Static Contact Angle

The contact angle of 8-Phe-4, PEA-*b*-PCL (PEA was from 8-Phe-4 with M_n of 4,000, and the feed weight ratio of 8-Phe-4/ ϵ -CL = 1:6), and PCL were measured and compared (Table 6.6). It was found that all three types of polymers showed high water contact angles (around or above 80 degree). The contact angle is the angle formed by a liquid at the three phase boundary where the liquid, gas, and solid intersect. The small contact angle means that the adhesive forces are dominating, while the high contact angle means the cohesive forces are dominating. Based on the obtained contact angle data, the introduction of PEA into the PCL backbone brought about 10 % reduction in wettability from pure PCL (from 90.86 to 81.58°). The contact angle of the 8-Phe-4-*b*-PCL, however, is only marginally higher than the pure 8-Phe-4.

Table 6.4 Information of some PEA-*b*-PCLs

PEA	M _n (KDa)	PEA/ ϵ -CL (w/w)	M _n (theoretical)	M _n (measured)	PDI	Yield (%)
8-Phe-4	4.1	1:1	8.2	7.7	1.42	85
8-Phe-4	4.1	1:3	16.4	17.2	1.55	87
8-Phe-4	4.1	1:6	28.4	31.6	1.37	93
8-Phe-4	4.1	1:8	36.9	32.0	1.36	81
8-Phe-4	7.7	1:1	15.4	14.7	1.45	85
8-Phe-4	7.7	1:2	23.1	23.7	1.41	91
8-Phe-4	7.7	1:3	30.8	28.8	1.59	89
8-Phe-4	7.7	1:6	53.9	45.6	1.50	94
PCL	80.0	NA	NA	NA	NA	NA

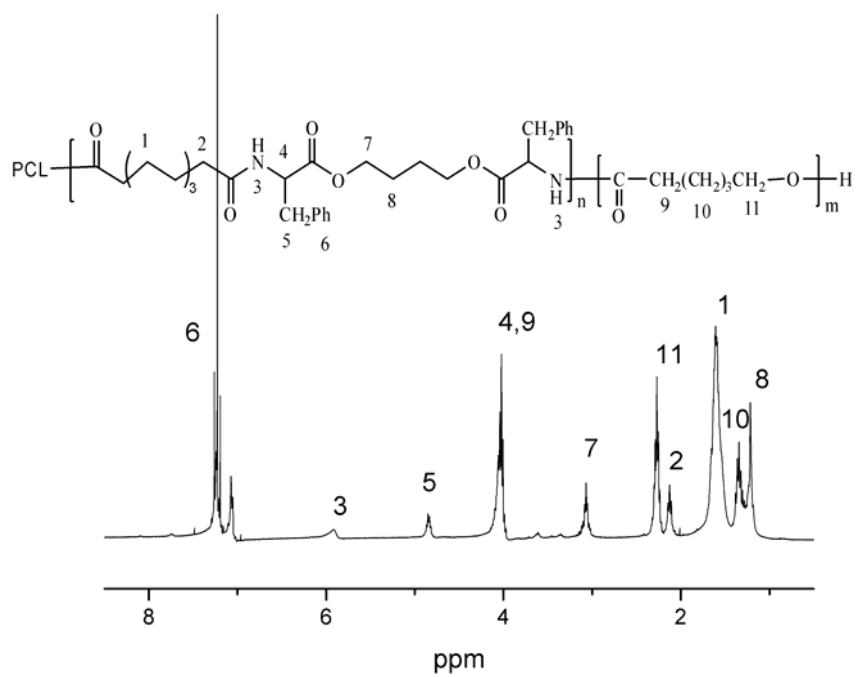


Figure 6.6 ^1H -NMR spectrum of 8-Phe-4-*b*-PCL synthesized from 8-Phe-4 of M_n 4,000 and 8-Phe-4/ ϵ -CL=1.0:1.0, w/w)

Table 6.5 Solubility of PEA-*b*-PCLs*

PEA- <i>b</i> -PCL MW	Ethyl ether	Ethanol	Acetone	THF	Chloroform	DMF	DMSO
7.7	-	-	-	+	+	+	+
17.2	-	-	-	+	+	+	+
31.6	-	-	-	+	+	+	+
PCL	-	-	+	+	+	+	+
8-Phe-4	-	-	-	+	+	+	+

* The PEA-*b*-PCLs are all 8-Phe-4-*b*-PCL (M_n of 8-Phe-4 is around 4,000; + means soluble, - means insoluble)

Table 6.6 static contact angle of polymers

Polymer Name	PCL	8-Phe-4	PEA- <i>b</i> -PCL(1)
Static Angle(°)	90.86±0.77	79.62±1.03	81.58±0.94

6.D.5 Thermal Property of PEA-*b*-PCLs

The reported melting temperature (T_m) and glass transition temperature (T_g) of pure PCL are around 64-65 °C and -60 °C respectively. For pure Phe-based PEA of M_n 25,000-30,000, such as 8-Phe-4, the T_m and T_g are around 111 °C and 47 °C respectively²⁷. For the 8-Phe-4-*b*-PCL, no obvious T_g peaks could be detected in the DSC scanning range from -30 °C to 150 °C.

It was found that PEA-*b*-PCL could have 2 T_m peaks from PCL part and PEA part respectively. And the T_m values for PEA (8-Phe-4) part are: 98-100 °C for PEA with M_n around 4,000; and 110-112 °C for PEA (8-Phe-4) with M_n around 7-9,000. The thermal data indicated that the T_m values of PEA (8-Phe-4) part are mainly affected by the M_n of PEA. However, the T_m peak from the PCL part was found that it was significantly affected by the copolymer composition. Figure 6.7 and Table 7 showed an example that how the block copolymer composition affected the T_m of PCL part. Pure PCL and 3 types of PEA-*b*-PCL were selected for T_m comparison. All of the PEA-*b*-PCL were 8-Phe-4-*b*-PCL and M_n of 8-Phe was 4.1k. The only difference is that the weight ratio of ϵ -CL/8-Phe-4 was from 3.0 to 6.0 and 8.0. The data in Figure 6.7 show that the T_m value of the PCL part in the 8-Phe-4-*b*-PCL copolymer increased with an increase in the feed weight ratio of ϵ -CL/8-Phe-4 and this composition effect on the T_g is consistent with the reported polyester systems³⁵.

6.D.6 Formulation of PEA-*b*-PCL into Microspheres and Electrospun Fibers

In order to test the processing capability of these newly synthesized PEA-*b*-PCL copolymers, two fabrications methods, microspheres and electrospun micro/nano fibers, were used. Figure 6.8 is an example for the PEA-*b*-PCL (PEA was from 8-Phe-4 with M_n of 4,000, and the feed weight ratio of 8-Phe-4/ ϵ -CL = 1:6) microspheres with a diameter around 1-2 μ m. For the microsphere fabrication, the following different parameters were examined: polymer concentration, PVA concentration, and homogenizer speed³⁶. The optimized conditions for the PEA-*b*-PCL microspheres are: 1 wt% PVA in 100 mL water; 10 wt% polymer in 10 mL CHCl_3 ; homogenizer speed: 10,000 rpm for 5-8 min. PVA and polymer concentration could affect the microsphere size, while lower speeds or longer time may reduce the yield or cause the aggregation.

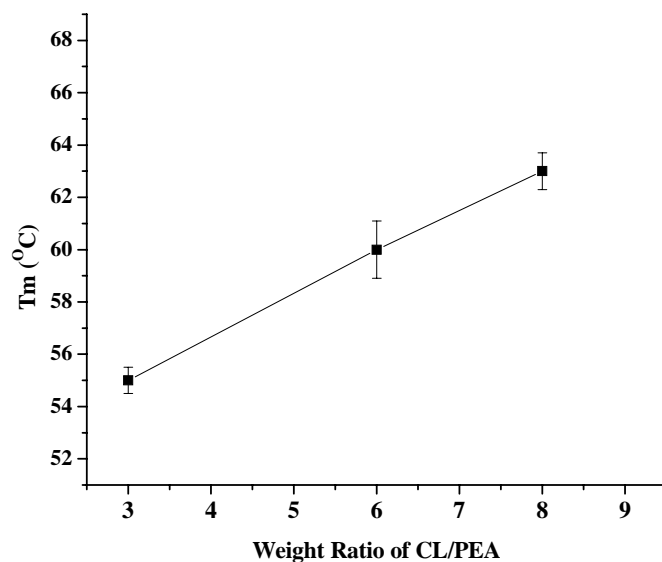


Figure 6.7 the effect of the ϵ -caprolactone (CL) to PEA feed ratio on the melting temperature (T_m) of PEA-*b*-PCL copolymers

Table 6.7 T_m and T_g of polymers

Polymer Name	T_m	T_g
PCL	64-65 °C	-60 °C
8-Phe-4	111°C	47 °C
8-Phe-4- <i>b</i> -PCL(1/3,w/w)	55 °C	NA
8-Phe-4- <i>b</i> -PCL(1/6,w/w)	60 °C	NA
8-Phe-4- <i>b</i> -PCL(1/8,w/w)	63 °C	NA

Note: The thermal data of PCL³⁷ and 8-Phe-4²⁷ are from the references.

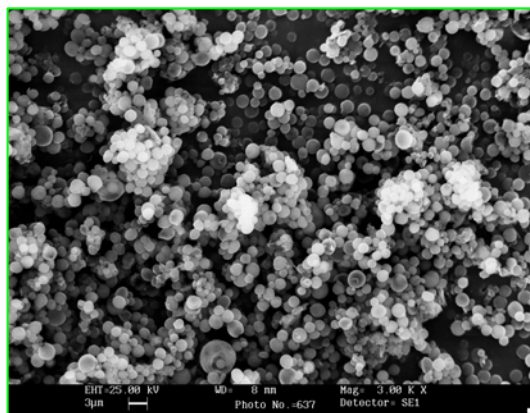


Figure 6.8 SEM image of PEA-*b*-PCL microspheres. The PEA was from 8-Phe-4 with M_n 4,000, and the feed weight ratio of 8-Phe-4/ ϵ -CL was 1:6

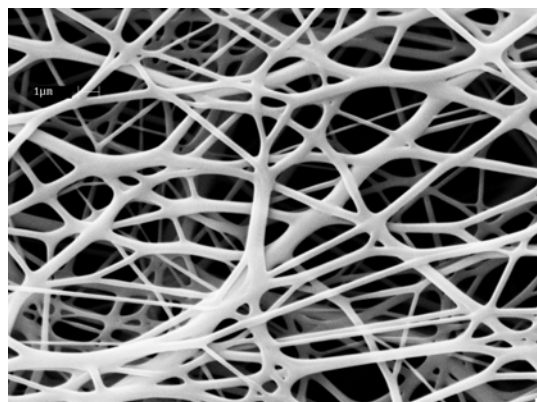


Figure 6.9 SEM image of PEA-*b*-PCL electrospun fibers. The PEA was from 8-Phe-4, of M_n 4,000, and the feed weight ratio of 8-Phe-4/ ϵ -CL was 1:6

Figure 6.9 is an example for the PEA-*b*-PCL electrospun micro/nano fibers with a fiber diameter around 0.3-1 μm . The fiber fabrication conditions were also optimized³⁸. From Figures 6.8 and 6.9, we could state the new PEA-*b*-PCL copolymers could be fabricated into different physical forms.

6.D.7 In vitro Enzymatic Biodegradation of PEA-*b*-PCL

Four types of polymer films were selected for the enzymatic biodegradation study: 8-Phe-4 ($M_n=30,000$), PCL ($M_n=80,000$), 8-Phe-4-*b*-PCL (M_n of 8-Phe-4: 4,000; PEA/ ϵ -CL=1:3, w/w), 8-Phe-4-*b*-PCL (M_n of 8-Phe-4: 4,000; PEA/ ϵ -CL=1:6, w/w). Figure 6.10 showed the enzymatic biodegradation results of the 4 polymers during the one month period. The weight loss data show that the pure PCL film as expected did not biodegrade during the 1 month period, while the 8-Phe-4 film was completely biodegraded within 2 weeks and the linear biodegradation curve suggested that the biodegradation mechanism of 8-Phe-4 was of surface erosion mechanism. For the 2 types of 8-Phe-4-*b*-PCL, both of them showed smaller weight loss than the pure 8-Phe-4, but higher than the pure PCL. The 8-Phe-4-*b*-PCL copolymer having a higher PCL component (i.e., PEA/ ϵ -CL=1:6, w/w), the block copolymer film showed almost no degradation, just like the pure PCL. With relatively more PEA component (PEA/ ϵ -CL=1:3, w/w); the block copolymer film showed almost the same weight loss pattern as the pure PCL. According to the previous publications^{27, 28, 39-41}, the Phe-PEA could be biodegraded by α -chymotrypsin within a few days or several weeks, and the degradation rate depends on the enzyme concentration and the physical form of the PEAs (powder, particle or solid film). The weight loss data also showed that an introduction of PCL into PEA did significantly alter the enzymatic biodegradation property of PEA, and the resulting PEA-*b*-PCL's degradation property became closer to PCL, and the level of change depended on the PEA to PCL feed ratio during the copolymer synthesis.

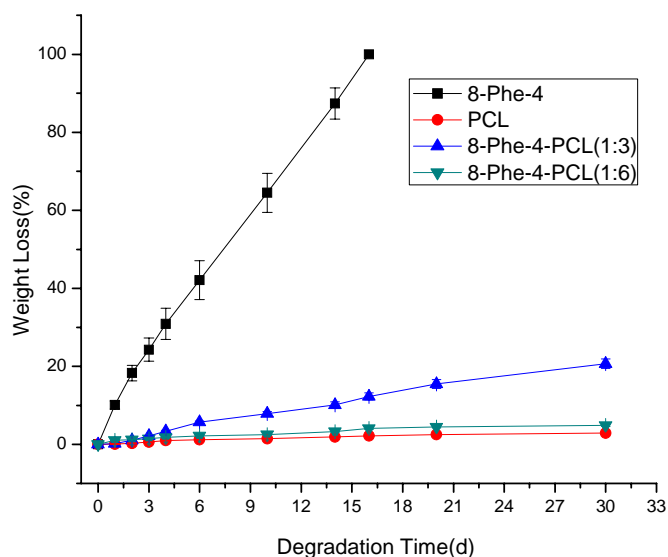


Figure 6.10 Effect of PEA to PCL feed ratio on the enzymatic biodegradation of the PEA-*b*-PCL copolymers. Pure PEA and PCL served as the controls. 8-Phe-4 ($M_n=30,000$), PCL ($M_n=80,000$), 8-Phe-4-*b*-PCL (M_n of 8-Phe-4: 4,000; PEA/ ϵ -CL=1:3, w/w), 8-Phe-4-*b*-PCL (M_n of 8-Phe-4: 4,000; PEA/ ϵ -CL=1:6, w/w). Enzyme solution is 0.2 mg/mL of α -chymotrypsin in PBS solution.

6.D.8 Cell Attachment and Proliferation Assays

Six types of polymers and their copolymers and mixtures were tested here. They are: pure PCL, PEA/PCL (8-Phe-4 with M_n of 4,000; at the mixing ratio of 1:3; w/w), PEA/PCL (8-Phe-4 with M_n of 4,000; at the mixing ratio of 1:6; w/w), PEA-*b*-PCL (8-Phe-4 with M_n of 4,000, PEA/ ϵ -CL=1:3; w/w), PEA-*b*-PCL (8-Phe-4 with M_n of 4,000, PEA/ ϵ -CL=1:6; w/w), and pure PEA (8-Phe-4, $M_n=30,000$). The pure PEA and PCL were used as the controls. From microscopic cell morphology images (Figure 9), it was found that after coating on the glass coverslips, the mixture of PEA/PCL could not form a good coating with smooth or well organized surface. Meanwhile, the pure

PEA or PCL could form a good and smooth coating. For the PEA-*b*-PCL, the copolymer coating showed some organized microstructure, which could be due to the self-assembly happened during the drying process. The self-assembly of the copolymers was induced because of the chemical difference of the PEA and PCL segments toward dissolution and drying processes. This surface microstructure of the PEA-*b*-PCL copolymers had been confirmed by many repeated trials.

The data in Figure 6.11 and the MTT assay (Figure 6.12) show that this self-assembly of PEA-*b*-PCL copolymers did not affect BAEC cell attachment/proliferation. Figure 6.11 showed that the PEA-*b*-PCL copolymers could significant increase cell attachment and proliferation when compared to pure PCL, almost reaching the same level as pure Phe-PEA. For PCL, the cell attachment was not as good as pure 8-Phe-4 and other coatings. For the PEA/PCL mixture, the cell attachment was poor when comparing to PEA-*b*-PCL. According to the previous reports, many types of PEA coatings could promote the cell attachment and proliferation^{30, 42-44}, while pure PCL was not a very good material for this purpose⁴⁵⁻⁵⁰. For example, Amato et al reported the poor human osteoblasts attachment on pure PCL surface, while the introduction of functional groups, such as amine groups, could significantly improve the human osteoblasts attachment performance on modified PCL surface.⁵⁰ The above results indicated that the cell attachment and proliferation performance of PCL could be significantly improved by introducing the PEA segments into PCL chain. The cell data also showed that the PEA-*b*-PCL copolymers have totally different cellular response from the PEA/PCL mixture. These data suggest that a simple coating of the PEA/PCL mixture could not improve the cellular response of PCL.

A comparison of the cell data among PEA-*b*-PCL, pure PCL and pure PEA shows that the cell data are consistent with the contact angle data (Table 6). For the pure PCL, the contact angle is around 91 °, while 8-Phe-4 and 8-Phe-4 have the contact angles of 79 ° and 81 °, respectively. 8-Phe-4 has showed excellent cell attachment and proliferation performance. The chemical incorporation of 8-Phe-4 into PCL significantly changed the surface property of PCL (i.e., reduce its contact angle from 91° to 81°), and significantly improved the cell attachment and proliferation performance of PCL. The 8-Phe-4-*b*-PCL has a similar contact angle as the pure 8-Phe-4, so it's not surprising that both polymers showed similar good cell attachment and proliferation performance.

For the proliferation tests, an increase in cell number results in an increase in the amount of MTT formazan formed and an increase in UV absorbance. Figure 6.12 showed that the PCL and the PEA/PCL mixture could not support the bovine aortic endothelial cell (BAEC) proliferation well, while PEA-*b*-PCL and pure PEA could support the cell proliferation pretty well, and the PEA-*b*-PCL copolymers showed almost the same proliferated cell # as the pure PEA did. Thus, the chemically incorporation of PEA into PCL could significantly promote the cell proliferation of PCL-based biomaterials.

6.D.9 In Vitro Inflammatory Response of PEA-*b*-PCL

In this study, macrophage activation after 48 h incubation was measured using an ELISA kit to test mouse TNF- α production so that a quantitative inflammatory response could be determined. The PEA-*b*-PCL copolymer tested was synthesized from 8-Phe-4 with M_n of 4,000 at the PEA/ ϵ -CL=1:6; w/w. PMBA was used here as a

control because it's a widely used FDA approved polymer in medical devices like drug-eluting stents. The *in vitro* inflammatory data show that PEA-*b*-PCL copolymer had a significantly lower level inflammation response than PBMA and PCL control. For example, from Figure 6.13, the PEA-*b*-PCL show only 40 pg/mL TNF- α production by J774 mouse macrophages from 150 pg/mL TNF- α of a pure PCL, a reduction to less than 1/3 of the original TNF- α production of a pure PCL. For the pure PCL, Ainslie et al reported the PCL films would exhibit inflammatory response, although less than the lipopolysaccharide (LPS) ⁵¹. Others reported that the chemical modification of PCL, such as PLA-*b*-PCL-*b*-PEG, could decrease the PCL inflammation response to moderate level⁵² In this report, we show the PEA modification of PCL (PEA-*b*-PCL copolymer) also significantly reduced the inflammation of pure PCL. It is important to know that the inflammatory data in Figure 6.13 also indicate that the PEA incorporated as a co-monomer unit into a copolymer has the capability to tame the inflammatory response induced by other relatively inflammatory biomaterials like PCL in this study. The very low inflammatory response to pure PEA-based biomaterials was also reported in other *in vitro* and *in vivo* studies^{44, 53-55}. Therefore, PEAs not only show very low level of inflammation by themselves but also could tame the inflammatory response induced by other biomaterials when PEAs are conjugated with other biomaterials. This unique biological property may have important impact on the developing low inflammatory biomaterials and medical devices.

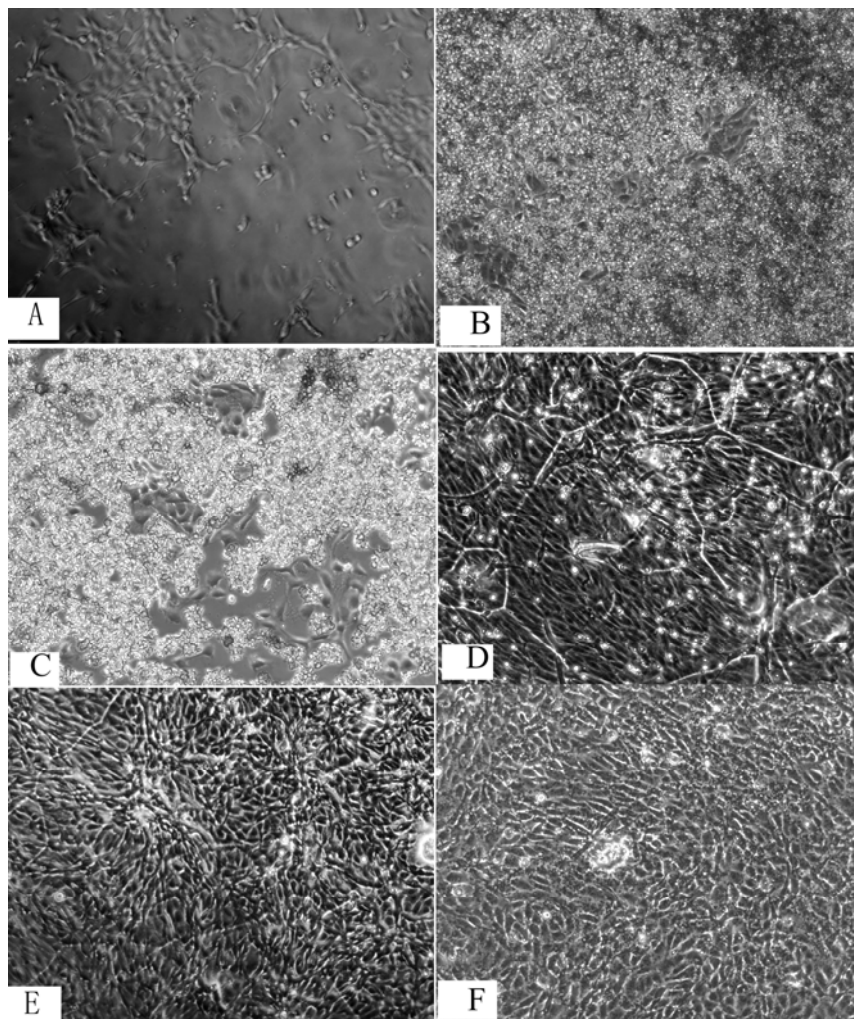


Figure 6.11 Microscopic Image of attached BAEC on the polymer coatings on glass coverslips for 96h: (A) PCL; (B) PEA/PCL mixture (1:3; w/w) (PEA: 8-Phe-4, $M_n=30,000$); (C) PEA/PCL mixture (1:6; w/w) (PEA: 8-Phe-4, $M_n=30,000$); (D) PEA-*b*-PCL copolymer (PEA/ ϵ -CL=1:3; w/w) (PEA: 8-Phe-4, $M_n=4,000$); (E) PEA-*b*-PCL copolymer (PEA/ ϵ -CL=1:6; w/w) (PEA: 8-Phe-4, $M_n=4,000$); (F) PEA (8-Phe-4, $M_n=30,000$)

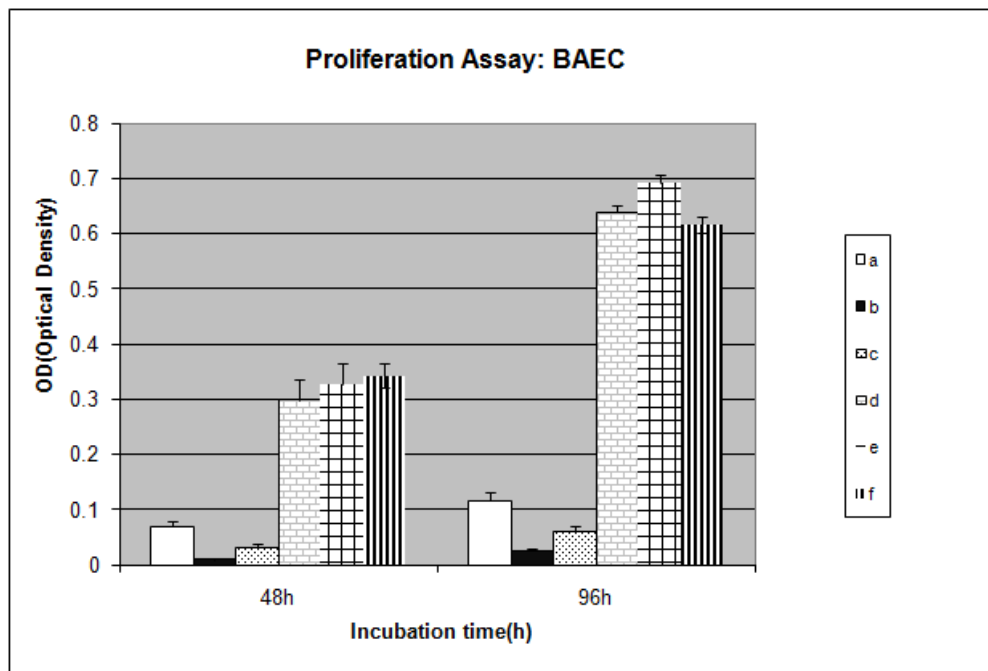


Figure 6.12 MTT proliferation assay of BAEC on polymer coatings on glass coverslips (a) PCL; (b) PEA/PCL mixture (1:3; w/w) (PEA: 8-Phe-4, $M_n=30,000$);(c) PEA/PCL mixture (1:6;w/w) (PEA: 8-Phe-4, $M_n=30,000$); (d) PEA-*b*-PCL copolymer (PEA/ε-CL=1:3; w/w) (PEA: 8-Phe-4, $M_n=4,000$); (e) PEA-*b*-PCL copolymer (PEA/ε-CL=1:6; w/w) (PEA: 8-Phe-4, $M_n=4,000$); (f) PEA (8-Phe-4, $M_n=30,000$)

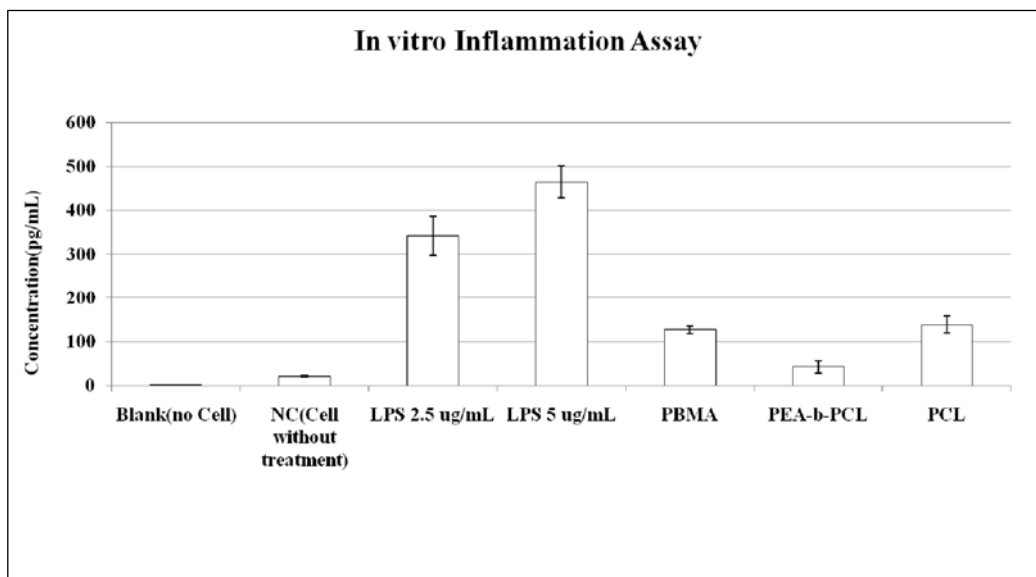


Figure 6.13 In vitro Measurement of Inflammatory Response of Polymers. J774 mouse macrophages were seeded on the polymer film to interact with the polymers. The mouse TNF- α production concentration was measured as an index of the inflammatory response. Blank control has no cells and negative control (NC) has the J774 mouse macrophages without any polymer treatment. LPS is the lipopolysaccharide.

6.E Conclusion

A new biodegradable block copolymer family, PEA-*b*-PCL, has been successfully prepared and characterized. In a certain range, the molecular weight and polymer structure of PEA-*b*-PCLs could be well estimated and controlled. PEA-*b*-PCLs could be easily fabricated into different formulations, such as microspheres and electrospun fibers. Enzyme degradation tests showed that introducing PEA to PCL did not significantly change the degradation behavior of PCL. Cell attachment and proliferation tests showed that the PEA segment in the PEA-*b*-PCL could greatly

increase the cell attachment and proliferation performance of PCL, and could reach the same level as the PEA. The in vitro inflammation response test also proved that the PEA-*b*-PCL had low inflammation response. So the PEA-*b*-PCLs combined the favorable properties of PCL and PEA and expand the applications in the biomedical and pharmaceutical areas. The synthesis route of PEA-*b*-PCL could be easily applied to other polyester systems to obtain a variety of PEA-*b*-Polyesters.

REFERENCE

1. Uhrich, K. E.; Cannizzaro, S. M.; Langer, R. S.; Shakesheff, K. M., Polymeric Systems for Controlled Drug Release. *Chem. Rev. (Washington, D. C.)* **1999**, 99, (11), 3181-3198.
2. Gref, R.; Minamitake, Y.; Peracchia, M. T.; Trubetskoy, V.; Torchilin, V.; Langer, R., Biodegradable long-circulating polymeric nanospheres. *Science* **1994**, 263, (5153), 1600-3.
3. Langer, R., New methods of drug delivery. *Science (New York, N.Y.)* **1990**, 249, (4976), 1527-33.
4. Langer, R., Drug delivery and targeting. *Nature (London)* **1998**, 392, (6679, Suppl.), 5-10.
5. Langer, R. S.; Peppas, N. A., Present and future applications of biomaterials in controlled drug delivery systems. *Biomaterials* **1981**, 2, (4), 201-14.
6. Chu, C.-C., Biodegradable polymeric biomaterials: an updated overview. *Biomaterials* **2007**, 6/1-6/22.
7. Zhu, A.; Zhang, M.; Wu, J.; Shen, J., Covalent immobilization of chitosan/heparin complex with a photosensitive hetero-bifunctional crosslinking reagent on PLA surface. *Biomaterials* **2002**, 23, (23), 4657-4665.
8. Bordes, P.; Pollet, E.; Averous, L., Nano-biocomposites: Biodegradable polyester/nanoclay systems. *Progress in Polymer Science* **2009**, 34, (2), 125-155.
9. Coulembier, O.; Degee, P.; Hedrick, J. L.; Dubois, P., From controlled ring-opening polymerization to biodegradable aliphatic polyester: Especially poly(beta - malic acid) derivatives. *Progress in Polymer Science* **2006**, 31, (8), 723-747.
10. Gross, R. A.; Kalra, B., Biodegradable polymers for the environment. *Science (Washington, DC, United States)* **2002**, 297, (5582), 803-807.

11. Maharana, T.; Mohanty, B.; Negi, Y. S., Melt-solid polycondensation of lactic acid and its biodegradability. *Progress in Polymer Science* **2009**, 34, (1), 99-124.
12. McKee, M. G.; Unal, S.; Wilkes, G. L.; Long, T. E., Branched polyesters: recent advances in synthesis and performance. *Progress in Polymer Science* **2005**, 30, (5), 507-539.
13. Okada, M., Chemical syntheses of biodegradable polymers. *Progress in Polymer Science* **2001**, 27, (1), 87-133.
14. Varma, I. K.; Albertsson, A.-C.; Rajkhowa, R.; Srivastava, R. K., Enzyme catalyzed synthesis of polyesters. *Progress in Polymer Science* **2005**, 30, (10), 949-981.
15. West, J. L., Drug delivery: Pulsed polymers. *Nature Materials* **2003**, 2, (11), 709-710.
16. Wu, J.; Wang, X.; Keum, J. K.; Zhou, H.; Gelfer, M.; Avila-Orta, C.-A.; Pan, H.; Chen, W.; Chiao, S.-M.; Hsiao, B. S.; Chu, B., Water soluble complexes of chitosan-g-MPEG and hyaluronic acid. *Journal of Biomedical Materials Research, Part A* **2007**, 80A, (4), 800-812.
17. Huang, N. F.; Patel, S.; Thakar, R. G.; Wu, J.; Hsiao, B. S.; Chu, B.; Lee, R. J.; Li, S., Myotube Assembly on Nanofibrous and Micropatterned Polymers. *Nano Letters* **2006**, 6, (3), 537-542.
18. Zhu, K. J.; Lin, X.; Yang, S., Preparation, characterization, and properties of polylactide (PLA)-poly(ethylene glycol) (PEG) copolymers: a potential drug carrier. *Journal of Applied Polymer Science* **1990**, 39, (1), 1-9.
19. Gref, R.; Minamitake, Y.; Peracchia, M. T.; Trubetskoy, V.; Torchilin, V.; Langer, R., Biodegradable long-circulating polymer nanospheres. *Science (Washington, DC, United States)* **1994**, 263, (5153), 1600-3.

20. Perez, C.; Sanchez, A.; Putnam, D.; Ting, D.; Langer, R.; Alonso, M. J., Poly(lactic acid)-poly(ethylene glycol) nanoparticles as new carriers for the delivery of plasmid DNA. *Journal of Controlled Release* **2001**, 75, (1-2), 211-224.
21. Hwang, M. J.; Suh, J. M.; Bae, Y. H.; Kim, S. W.; Jeong, B., Caprolactonic poloxamer analog: PEG-PCL-PEG. *Biomacromolecules* **2005**, 6, (2), 885-890.
22. Bae, S. J.; Joo, M. K.; Jeong, Y.; Kim, S. W.; Lee, W.-K.; Sohn, Y. S.; Jeong, B., Gelation Behavior of Poly(ethylene glycol) and Polycaprolactone Triblock and Multiblock Copolymer Aqueous Solutions. *Macromolecules* **2006**, 39, (14), 4873-4879.
23. Barrera, D. A.; Zylstra, E.; Lansbury, P. T., Jr.; Langer, R., Synthesis and RGD peptide modification of a new biodegradable copolymer: poly(lactic acid-co-lysine). *Journal of the American Chemical Society* **1993**, 115, (23), 11010-11.
24. Barrera, D. A.; Zylstra, E.; Lansbury, P. T.; Langer, R., Copolymerization and Degradation of Poly(lactic acid-co-lysine). *Macromolecules* **1995**, 28, (2), 425-32.
25. Wang, Y.; Ameer, G. A.; Sheppard, B. J.; Langer, R., A tough biodegradable elastomer. *Nature Biotechnology* **2002**, 20, (6), 602-606.
26. Langer, R.; Tirrell, D. A., Designing materials for biology and medicine. *Nature (London, United Kingdom)* **2004**, 428, (6982), 487-492.
27. Katsarava, R.; Beridze, V.; Arabuli, N.; Kharadze, D.; Chu, C. C.; Won, C. Y., Amino acid-based bioanalogous polymers. synthesis, and study of regular poly(ester amide)s based on bis(alpha -amino acid) alpha ,w-alkylene diesters, and aliphatic dicarboxylic acids. *Journal of Polymer Science, Part A: Polymer Chemistry* **1999**, 37, (4), 391-407.
28. Guo, K.; Chu, C. C., Synthesis, characterization, and biodegradation of copolymers of unsaturated and saturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2007**, 45, (9), 1595-1606.

29. Guo, K.; Chu, C. C.; Chkhaidze, E.; Katsarava, R., Synthesis and characterization of novel biodegradable unsaturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2005**, 43, (7), 1463-1477.
30. Deng, M.; Wu, J.; Reinhart-King, C. A.; Chu, C.-C., Synthesis and Characterization of Biodegradable Poly(ester amide)s with Pendant Amine Functional Groups and in Vitro Cellular Response. *Biomacromolecules* **2009**, 10, (11), 3037-3047.
31. Yamanouchi, D.; Wu, J.; Lazar, A. N.; Craig Kent, K.; Chu, C.-C.; Liu, B., Biodegradable arginine-based poly(ester-amide)s as non-viral gene delivery reagents. *Biomaterials* **2008**, 29, (22), 3269-3277.
32. Huang, S. J.; Bansleben, D. A.; Knox, J. R., Biodegradable polymers: Chymotrypsin degradation of a low molecular weight poly(ester-urea) containing phenylalanine. *Journal of Applied Polymer Science* **1979**, 23, (2), 429-37.
33. Bhatia, S. K.; Arthur, S. D., Poly(vinyl alcohol) acetoacetate-based tissue adhesives are non-cytotoxic and non-inflammatory. *Biotechnol. Lett.* **2008**, 30, (8), 1339-1345.
34. Guo, K.; Chu, C. C., Synthesis and characterization of novel biodegradable unsaturated poly(ester amide)/poly(ethylene glycol) diacrylate hydrogels. *Journal of Polymer Science, Part A: Polymer Chemistry* **2005**, 43, (17), 3932-3944.
35. Castillo, R. V.; Muller, A. J.; Raquez, J.-M.; Dubois, P., Crystallization Kinetics and Morphology of Biodegradable Double Crystalline PLLA-b-PCL Diblock Copolymers. *Macromolecules (Washington, DC, U. S.)* 43, (9), 4149-4160.
36. Guo, K.; Chu, C. C., Biodegradable and injectable paclitaxel-loaded poly(ester amide)s micropheres: fabrication and characterization. *Journal of Biomedical Materials Research, Part B: Applied Biomaterials* **2009**, 89B, (2), 491-500.

37. Tanaka, T.; Tsuchiya, T.; Takahashi, H.; Taniguchi, M.; Lloyd, D. R., Microfiltration membrane of polymer blend of poly(L-lactic acid) and poly(ε-caprolactone). *Desalination* **2006**, 193, (1-3), 367-374.
38. Li, L.; Chu, C.-C., Nitroxyl radical incorporated electrospun biodegradable poly(ester amide) nanofiber membranes. *Journal of Biomaterials Science, Polymer Edition* **2009**, 20, (3), 341-361.
39. Chu, C. C., Biodegradable hydrogels as drug controlled release vehicles. *Tissue Engineering and Novel Delivery Systems* **2004**, 423-461.
40. Tsitlanadze, G.; Kviria, T.; Katsarava, R.; Chu, C. C., In vitro enzymatic biodegradation of amino acid based poly(ester amide)s biomaterials. *Journal of Materials Science: Materials in Medicine* **2004**, 15, (2), 185-190.
41. Tsitlanadze, G.; Machaidze, M.; Kviria, T.; Djavakhishvili, N.; Chu, C. C.; Katsarava, R., Biodegradation of amino-acid-based poly(ester amide)s: In vitro weight loss and preliminary in vivo studies. *Journal of Biomaterials Science, Polymer Edition* **2004**, 15, (1), 1-24.
42. Karimi, P.; Rizkalla, A. S.; Mequanint, K., Versatile biodegradable poly(ester amide)s derived from α-amino acids for vascular tissue engineering. *Materials* **3**, 2346-2368.
43. Knight, D. K.; Atkins, K. M.; De Wit, M. A.; Wang, Z.; Lopez, D.; Mequanint, K.; Gillies, E. R., Functionalized poly(ester amide)s as scaffolds for vascular tissue engineering. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* **51**, (1), 37-38.
44. De Fife, K. M.; Grako, K.; Cruz-Aranda, G.; Price, S.; Chantung, R.; MacPherson, K.; Khoshabeh, R.; Gopalan, S.; Turnell, W. G., Poly(ester amide) copolymers promote blood and tissue compatibility. *Journal of Biomaterials Science, Polymer Edition* **2009**, 20, (11), 1495-1511.

45. Alvarez-Perez, M. A.; Guarino, V.; Cirillo, V.; Ambrosio, L., Influence of Gelatin Cues in PCL Electrospun Membranes on Nerve Outgrowth. *Biomacromolecules* 11, (9), 2238-2246.
46. Drevelle, O.; Bergeron, E.; Senta, H.; Lauzon, M.-A.; Roux, S.; Grenier, G.; Faucheux, N., Effect of functionalized polycaprolactone on the behavior of murine preosteoblasts. *Biomaterials* 31, (25), 6468-6476.
47. Durgam, H.; Sapp, S.; Deister, C.; Khaing, Z.; Chang, E.; Luebben, S.; Schmidt, C. E., Novel degradable co-polymers of polypyrrole support cell proliferation and enhance neurite out-growth with electrical stimulation. *J. Biomater. Sci., Polym. Ed.* 21, (10), 1265-1282.
48. Rai, B.; Lin, J. L.; Lim, Z. X. H.; Guldberg, R. E.; Hutmacher, D. W.; Cool, S. M., Differences between in vitro viability and differentiation and in vivo bone-forming efficacy of human mesenchymal stem cells cultured on PCL-TCP scaffolds. *Biomaterials* 31, (31), 7960-7970.
49. Ruckh, T. T.; Kumar, K.; Kipper, M. J.; Popat, K. C., Osteogenic differentiation of bone marrow stromal cells on poly(ϵ -caprolactone) nanofiber scaffolds. *Acta Biomater.* 6, (8), 2949-2959.
50. Amato, I.; Ciapetti, G.; Pagani, S.; Marletta, G.; Satriano, C.; Baldini, N.; Granchi, D., Expression of cell adhesion receptors in human osteoblasts cultured on biofunctionalized poly-(ϵ -caprolactone) surfaces. *Biomaterials* **2007**, 28, (25), 3668-78.
51. Ainslie, K. M.; Tao, S. L.; Popat, K. C.; Daniels, H.; Hardev, V.; Grimes, C. A.; Desai, T. A., In vitro inflammatory response of nanostructured titania, silicon oxide, and polycaprolactone. *Journal of Biomedical Materials Research, Part A* **2009**, 91A, (3), 647-655.

52. Kang, Y. M.; Lee, S. H.; Lee, J. Y.; Son, J. S.; Kim, B. S.; Lee, B.; Chun, H. J.; Min, B. H.; Kim, J. H.; Kim, M. S., A biodegradable, injectable, gel system based on MPEG-b-(PCL-ran-PLLA) diblock copolymers with an adjustable therapeutic window. *Biomaterials* 31, (9), 2453-2460.
53. Gomurashvili, Z.; Zhang, H.; Jenkins, T. D.; Hughes, J.; Wu, M.; Lambert, L.; Eltepu, L.; Pabba, C.; Chowdari, N.; Vassilev, V.; Katsarava, R.; Turnell, W. G., From drug-eluting stents to biopharmaceuticals: poly(ester amide) a versatile new bioabsorbable biopolymer. *PMSE Prepr.* **2006**, 95, 826.
54. Horwitz, J. A.; Shum, K. M.; Bodle, J. C.; Deng, M.; Chu, C.-C.; Reinhart-King, C. A., Biological performance of biodegradable amino acid-based poly(ester amide)s: endothelial cell adhesion and inflammation in vitro. *J. Biomed. Mater. Res., Part A* **2010**, 95A, (2), 371-380.
55. Lee Seung, H.; Szinai, I.; Carpenter, K.; Katsarava, R.; Jokhadze, G.; Chu, C.-C.; Huang, Y.; Verbeken, E.; Bramwell, O.; De Scheerder, I.; Hong Mun, K., In-vivo biocompatibility evaluation of stents coated with a new biodegradable elastomeric and functional polymer. *Coron Artery Dis* **2002**, 13, (4), 237-41.

CHAPTER 7

WATER INSOLUBLE CATIONIC CHARGED BIODEGRADABLE POLY (ESTER
AMIDE)S: SYNTHESIS, CHARACTERIAZTION AND *IN VITRO* CELLULAR
RESPONSE

Jun Wu,[†] Chih-Chang Chu,^{†,‡}

[†] Department of Biomedical Engineering, Cornell University, Ithaca, NY 14853-4401,
USA

[‡] Department of Fiber Science and Apparel Design, Cornell University, Ithaca, NY
14853-4401, USA

7. A Abstract

In order to obtain a new type of positively charged and water insoluble polymer for biomedical applications, we reported the synthesis of a family of biodegradable L-arginine and L-phenylalanine based hybrid poly (ester amide)s (Arg-Phe-PEAs) by solution polycondensation. These new cationic PEA polymers consist of 3 nontoxic building blocks: natural amino acids (L-arginine and L-phenylalanine), diols, and dicarboxylic acids. All the prepared polymers were well characterized by standard physicochemical methods. Unlike the pure L-arginine based poly (ester amide)s (Arg-PEAs), which have very good water solubility, these hybrid Arg-Phe-PEAs are water insoluble due to the presence of L-phenylalanine. The static contact angle data showed that Arg-Phe-PEAs' surface are much more hydrophilic compared with the pure L-phenylalanine based poly (ester amide)s (Phe-PEAs). *In vitro* biological tests of Arg-Phe-PEAs included enzymatic biodegradation, cell attachment and proliferation, and macrophage inflammation tests. The enzymatic biodegradation tests showed that the biodegradation rate of Arg-Phe-PEAs could be well controlled by changing the polymer composition. The cell culture data indicated that the bovine aortic endothelial cells (BAEC) have very good cell attachment and proliferation on the Arg-Phe-PEA, almost same as the collagen coating. The *in vitro* inflammation tests showed that the new polymers have very low inflammation response. All these biological data suggest that these cationic and water insoluble Arg-Phe-PEAs could have great potential in the medical device coating, tissue engineering scaffolds and drug delivery. The synthesis route of Arg-Phe-PEAs could be easily applied to other amino acid based poly (ester amide) (PEA) systems to obtain a variety of water insoluble positively charged hybrid PEAs.

7.B Introduction

In recent years, due to the fast growing of biotechnology and pharmaceutical science, many new biodegradable and biocompatible polymers have been developed¹⁻⁸. Among them, biodegradable amino acids based poly (ester amide)s (PEAs, Figure 7.1) have been widely investigated due to their good biocompatibility, biodegradability and mechanical properties^{7, 9-20}. The amide and ester bonds on the PEA polymer backbone have provided merits of a combination of favorable properties of both polyesters and polyamides^{7, 9-21}. Biodegradable PEAs were usually synthesized by polycondensation reaction of α -amino acids, aliphatic dicarboxylic acids and diols¹¹. In the past few years, our group and other labs have established a general methodology for synthesis of PEAs through the solution polycondensation between di-*p*-toluenesulfonic acid salts of bis-(L- α -amino acid) α,ω -alkylene diesters (as bis-nucleophiles) and active di-*p*-nitrophenyl esters of dicarboxylic acids (as bis-electrophiles)^{7, 9-21}. By using this well-established method, we have successfully obtained many different types of saturated and unsaturated PEAs and their functional derivatives. These PEAs have also been engineered into a variety of physical forms, such as fibers²² and electrospun fibrous membranes²³, 3D microporous hydrogels^{17, 18} and microspheres²⁰. These PEAs appear to be good candidates for many biomedical applications ranging from surgical implants to drug/gene delivery and tissue engineering scaffolds^{7, 9-25}. The development of diverse applications for PEA has prompted us to design and prepare new functional PEAs.

Although PEAs consisting of different chemical structures have been synthesized and characterized by many research groups^{7, 9-28}, relatively little research efforts were focused on the synthesis of PEAs having pendant functional groups^{12, 24, 29}. The

introduction of pendant functional groups along the polymer backbone could provide an efficient method of not only synthesizing more PEA derivatives but also tailoring the properties of PEAs including hydrophilicity, charge property, biodegradation rate and mechanical strength. In addition, the pendant functional groups would permit for chemically conjugating biologically active agents for controlled delivery. One newly developed synthesis method to achieve functional PEAs was based on the copolymer of PEA, which could introduce free amine, carboxylic acid or hydroxyl groups^{12, 24, 25, 27-29}. Another newly reported example is to use acidic or basic amino acids, such as the cationic L-arginine (Arg) to synthesize Arg-based poly (ester amide) (Arg-PEA)^{15, 27, 28}. However, the good water solubility of Arg-PEA restricted its applications as the biomaterials for biomedical devices or surgical implants.

In order to expand the applications of Arg-PEAs, we are reporting a new family of water insoluble and cationic hybrid Arg-Phe-PEAs. This method involves the polycondensation reaction of the mixture consisting of 3 different monomers: di-*p*-toluenesulfonic acid diester salts of bis- (L-phenylalanine), α , ω -alkylene diesters/tetra-*p*-toluenesulfonic acid salts of bis- (L-arginine), and di-*p*-nitrophenyl ester of dicarboxylic acids. The percentage of L-arginine of the resulting PEAs could be controlled by the adjustment of the feed ratio of di-*p*-toluenesulfonic acid salts of bis- (L-phenylalanine) to tetra-*p*-toluenesulfonic acid salts of bis- (L-arginine). Compared with neutral pure Phe-PEAs, the new Arg-Phe-PEAs are positively charged. And compared with water soluble pure Arg-PEAs, the Arg-Phe-PEAs are water insoluble and can be processed in organic solvents. This approach could be a universal and simple synthetic route with a high potential utility for the preparation of hybrid PEAs having different pendant groups.

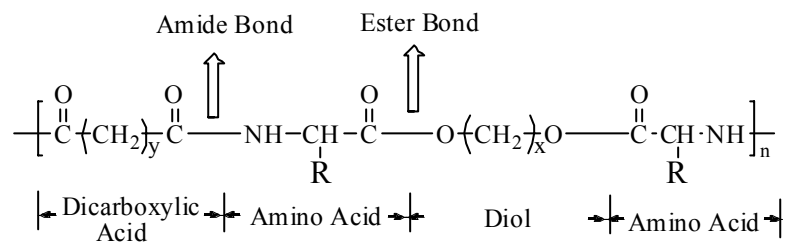


Figure 7.1 Chemical structure of poly (ester amide)

7.C Experimental

7.C.1 Materials

L-Arginine (L-Arg), L-phenylalanine (L-Phe), *p*-toluenesulfonic acid monohydrate, adipoyl chloride, sebacoyl chloride, 1, 4-butanediol, 1,6-hexanediol and *p*-nitrophenol were all purchased from Alfa Aesar (Ward Hill, MA) and used without further purification. Poly (n-butyl methacrylate) (PBMA) was purchased from Polysciences (Warrington, PA) and used directly. Triethylamine from Fisher Scientific (Fairlawn, NJ) was dried by refluxing with calcium hydride, and then distilled before use. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), Phosphate-buffered saline (PBS, 0.1M, pH 7.4) were purchased from Sigma (St. Louis, MO). Organic solvents like methanol, toluene, ethyl acetate, acetone, 2-propanol and dimethyl sulfoxide (DMSO) were purchased from VWR Scientific (West Chester, PA) and were purified by standard methods before use. Other chemicals and reagents if not otherwise specified were purchased from Sigma (St. Louis, MO). α -Chymotrypsin (Type II, from bovine pancreas, 66 units/mg, solid) was purchased from Sigma Chemical Co. (St. Louis, MO) and chosen as the model enzyme because it could hydrolyze ester linkages at C-terminal of hydrophobic α -amino acids like L-phenylalanine.

7.C.2 Synthesis of Monomers and Polymers

7.C.2.a Synthesis of Monomers

The general scheme of the synthesis of Arg-Phe-PEAs was divided into the following three major steps : the preparation of di-*p*-nitrophenyl ester of dicarboxylic acids (**I**) (Figure 7.2); the preparation of tetra-*p*-toluenesulfonic acid salts of bis (L-arginine), α , ω -alkylene diesters, and di-*p*-toluenesulfonic acid salts of bis- (L-phenylalanine), α , ω -alkylene diesters (**II**) (Figure 7.3); and the synthesis of Arg-Phe-PEAs (**III**) via solution polycondensation of (**I**) and (**II**) (Figure 7.4). All these three monomers have been synthesized in our prior studies ¹¹. Two types of monomer (**I**) were made: di-*p*-Nitrophenyl Adipate (**NA**), $x=4$; di-*p*-Nitrophenyl Sebacate (**NS**), $x=8$. x indicates the numbers of methylene group in the diacid. And four types of L-phenylalanine or L-arginine based monomers (**II**) were made in this study: di-*p*-toluenesulfonic acid salt of bis (L-phenylalanine) butane diesters, **Phe-4**, $y=4$; di-*p*-toluenesulfonic acid salt of bis (L-phenylalanine) hexane diesters, **Phe-6**, $y=6$; tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) butane diesters, **Arg-4-S**, $y=4$; tetra-*p*-toluenesulfonic acid salt of bis L-arginine) hexane diesters, **Arg-6-S**, $y=6$. S indicated that the L-arginine diester monomer was in the *p*-toluenesulfonic acid salt form.

7.C.2.b Synthesis of Arg-Phe-PEA (III) by Solution Polycondensation of (I) and (II) Monomers

Arg-Phe-PEAs were prepared by the solution polycondensation of the above (**I**) and (**II**) monomers (**Phe-4**, **Phe-6**, **Arg-4-S**, **Arg-6-S** and **NA**, **NS**) at different combinations and are listed in Table 7.1. These Arg-Phe-PEAs are labeled as x -Arg- y_1 -S- x -Phe- y_2 -Z%, where x and y_1/y_2 are the numbers of methylene groups in diacid

and diol, respectively, and $z\%$ is the molar percent of L-arginine diester monomer in the mixture of L-arginine diester monomer and L-phenylalanine diester monomer. For example, the 8-Arg-6-S-8-Phe-4-20% sample indicates that the x of this copolymer is 8, and y_1 (in Arg-block) is 6 and y_2 (in Phe-block) is 4, and the molar % of L-Arg diester monomer II in the copolymer is 20%.

In order to simplify the PEA system for study, x was fixed in each copolymer in this study, suggesting only one type of monomer **I**, NA or NS, was used for each polymer. y_1 is from arginine monomer **II** and y_2 is from phenylalanine monomer **II**. One type of Phe-PEA (8-Phe-4) was also prepared here as the polymer control and its synthesis steps were reported previously¹¹.

An example of the synthesis of 8-Arg-6-S-8-Phe-4-20% via a solution polycondensation is given here. Monomers NS (1.0 mmol), Arg-6-S (0.2 mmol) and Phe-4 (0.8 mmol) in 1.5 mL of dry DMSO were mixed well by vortexing. The mixture solution was heated up to 75 °C with stirring to obtain a uniformed mixture. Triethylamine (0.31 mL, 2.2 mmol) was added drop by drop to the mixture at 75 °C with vigorous stirring until a complete dissolution of the monomers. The solution color turned into yellow after several minutes. The reaction vial was then kept for 48 h at 75 °C in a thermostat oven without stirring. The polymer product was precipitated out from the solution by adding cold ethyl acetate, followed by decanted, dried and purified by Soxhlet extractor using ethyl acetate as solvent for 24 h. The final dried Arg-Phe-PEAs are yellow or pale yellow solid and dried *in vacuo* at room temperature.

7.C.3 Characterization

The physicochemical properties of the prepared monomers and polymers were characterized by various standard methods. For Fourier transform infrared (FTIR) characterization, the samples were ground into powders and mixed with KBr at a sample/KBr ratio of 1:10 (w/w). FTIR spectra were then obtained with a Perkin-Elmer (Madison, WI) Nicolet Magana 560 FTIR spectrometer with Omnic software for data acquisition and analysis. NMR spectra were recorded by a Varian Unity Inova 400-MHz spectrometer (Palo Alto, CA). Deuterated dimethyl sulfoxide (DMSO-*d*₆; Cambridge Isotope Laboratories) was used as the solvent. MestReNova software was used for the data analysis. The thermal properties were characterized with a DSC 2920 (TA Instruments, New Castle, DE). The measurements were carried out from -10 to 200 °C at a scanning rate of 10 °C/min and at a nitrogen gas flow rate of 25 mL/min. TA Universal Analysis software was used for thermal data analysis. The solubility of polymers in common organic solvents at room temperature was assessed by using 10.0 mg/mL as solubility criteria. The static contact angle of polymers was measured by a Ramé-Hart Model 500 Advanced Goniometer/Tensiometer. The round micro cover glasses (diameter, 12 mm, no.2, VWR, West Chester, PA) were coated with a polymer DMF solutions (2 wt%) and vacuum drying before testing. The static contact angle was measured by dropping distilled water (4 µL) onto the polymer coated cover glass surface. An average of triplicates was used.

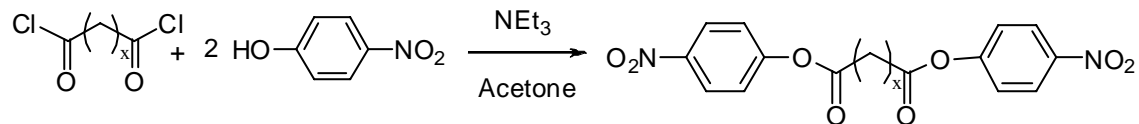


Figure 7.2 Synthesis of di-*p*-nitrophenyl ester of dicarboxylic acids monomer **I**.

Table 7.1 Synthesized Arg-Phe-PEAs

Monomer I	Monomer II (L-arginine)	Monomer II (L- Phenylalanine)	Molar percent of L-Arg Monomer II
NS	Arg-4-S	Phe-4	10 %
NS	Arg-4-S	Phe-4	20 %
NS	Arg-4-S	Phe-6	10 %
NS	Arg-4-S	Phe-6	20 %
NS	Arg-6-S	Phe-4	10 %
NS	Arg-6-S	Phe-4	20 %
NS	Arg-6-S	Phe-6	10 %
NS	Arg-6-S	Phe-6	20 %
NA	Arg-4-S	Phe-4	10 %
NA	Arg-4-S	Phe-4	20 %
NA	Arg-4-S	Phe-6	10 %
NA	Arg-4-S	Phe-6	20 %

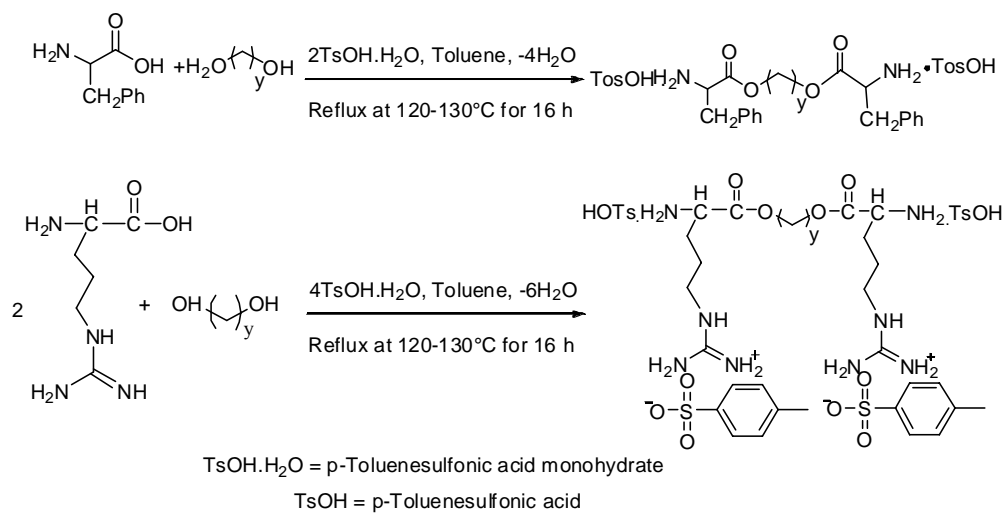
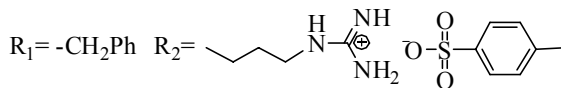


Figure 7.3 Synthesis of di-*p*-toluenesulfonic acid salt of bis (L-Phenylalanine) alkylene diesters and tetra-*p*-toluenesulfonic acid salt of bis(L-Arginine) alkylene diesters as monomers **II**.



and II at different feed ratios.

7.C.4 *In vitro* Enzymatic Biodegradation of Arg-Phe-PEA

The following polymers were selected for the *in vitro* enzymatic biodegradation tests. They were: 8-Arg-6-S-8-Phe-4-S-10%, 8-Arg-6-S-8-Phe-4-S-20% and 8-Phe-4. The polymer film samples (around 200 mg each, same size and thickness, round shape) was immersed in a small vial containing 10 mL of PBS buffer (pH= 7.4, 0.1 M) with or without α -chymotrypsin. The concentration of α -chymotrypsin was 0.2 mg/mL. The vials were then incubated at 37 °C with constant reciprocal shaking (ca. 100 rpm). The immersion media were refreshed every time and every 2 days in order to maintain the enzymatic activity. After predetermined immersion duration, the polymer film samples were removed by filtration, washed with distilled water for 3 times, and then dried in vacuum at 35 °C for 24 h to completely remove the residue water. The degree of biodegradation was estimated from the weight loss of the polymer film based on the following equation:

$$W_t (\%) = (W_o - W_t) / W_o \times 100$$

Where W_o is the original weight of the dry polymer sample before immersion, and W_t is the dry polymer sample weight after incubation for t hours/days (with or without enzyme). The averaged weight loss of three specimens was measured for each sample (N=3)

7.C.5 *Cell Culture*

The interaction of Arg-Phe-PEAs with cells was preliminarily studied to determine the level of cell attachment, proliferation and inflammation response. Bovine Endothelial Aorta Cells (BAECs) were used as the model cells for attachment and proliferation tests. BAECs were purchase from VEC Technologies and maintained at

37 °C in 5 % CO₂ in Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10 % Fetal Clone III (HyClone, Logan, UT), and 1 % each of penicillin–streptomycin, MEM amino acids (Invitrogen, Carlsbad, CA), and MEM vitamins (Mediatech, Manassas, VA). BAECs were used from passages 8–12. J774 mouse peritoneal macrophages were used as the model cells for in vitro inflammation response tests, which were obtained from ATCC and cultured at 37 °C in 5 % CO₂ in DMEM supplemented with 10 % FBS. J774s were used from passage 5-10. For all the cells, the cell media was changed every 2 days. Cells were grown to 70 % confluence before splitting or harvesting.

7.C.6 Cell Attachment and Proliferation Assay on Arg-Phe-PEA Coatings

The evaluation of the BAEC attachment and proliferation capability on the Arg-Phe-PEA surface was performed by cell attachment assay followed by a MTT assay. The round micro cover glasses (diameter, 12 mm, no.2,VWR, West Chester, PA) were coated with polymer DMF solution (2 wt%) and vacuum drying. The following polymers were tested: PBMA, 8-Arg-6-S-8-Phe-4-20% and Phe-PEA (8-Phe-4). Commercially available PBMA was used as the positive control. The reason why PBMA was used is that PBMA has been widely used in the stent coating and other medical devices by some companies.³⁰ Cell culture plate coated with collagen was used as negative control. After drying, the polymer coated glass coverslips were placed into cell culture plates and were sterilized for overnight under UV irradiation before use.

BAEC cells at an appropriate cell density concentration (20,000 cells/well) were seeded onto each test well in 24-well plates (BD Falcon™, polystyrene treated) and

then incubated in a 37 °C, 5 % CO₂ incubator. Cell media was changed every day. After the predetermined periods (48 h and 96 h), the cell culture plates were removed from the incubator. Cell morphology was recorded under a microscope. The media from the wells were then aspirated, and 0.5 mL fresh media were added to each well. After that, 40 µL of MTT solution (5 mg/mL) was subsequently added to each well, followed by 4 h incubation at 37 °C, 5 % CO₂. The cell culture medium was carefully removed and 400 µL of acidic isopropyl alcohol (with 0.1 M HCl) was added to dissolve the formed formazan crystal. The plate was slightly shaken for 30 mins and 100 µL solution was transferred from each well to a 96 well cell culture plate. Optical density (OD) of each well was measured at 570 nm (subtract background reading at 690 nm) by using a microplate reader. Triplicates were used in each experiment.

7.C.7 In Vitro Measurement of Inflammatory Response of Arg-Phe-PEA³⁰

J774 macrophages were seeded at 10,000 cells/well onto 12mm polymer-coated glass coverslips in 24-well tissue culture plates. A plain glass coverslip was used as a negative control. Positive controls were glass coverslips in media containing Lipopolysaccharide (LPS, from E. coli 0111:B4, Sigma-Aldrich, St. Louis, MO) at final concentrations of 1.25 µg/mL and 5 µg/mL. A plain glass coverslip in media alone was used as a cell-free negative control. 8-Arg-6-S-8-Phe-4-20% was selected for this test. PBMA and PCL were used as polymer control. Macrophage activation after 48 h incubation was measured using an ELISA kit to measure mouse TNF-α release (Invitrogen, Carlsbad, CA) according to the exact manufacturer's protocol. Sample TNF-α concentrations were calculated for a standard curve using a 4-parameter standard curve-fitting algorithm (Gen5 software, BioTek Instruments, Winooski, VT). N=3 for all samples. All samples and standards were read in

duplicate on a 96-well plate reader at 450 nm and referenced against a chromogen blank.

7.C.8 Statistics

Where appropriate, the data are presented as mean \pm standard deviation calculated over at least three data points. Significant differences compared to control groups were evaluated by unpaired Student's t-test or Dunnet test at p 0.05, and between more than two groups by Tukey's test with or without one-way ANOVA analysis of variance. JMP software (version 8.0, from SAS Company) was used for data analysis.

7.D Results and Discussion

7.D.1 Synthesis and Characterization of PEAs

In this study, six types of monomers [two monomers **I** (NA, NS) and four monomers **II** (Phe-4, Phe-6, Arg-4-S and Arg-6-S)] were synthesized according to our previously published procedures¹¹ for preparing Arg-Phe-PEAs. All these monomers were prepared with high yields and easily purified by re-crystallization. The chemical structure and purity of these 6 monomers had been confirmed by ¹H-NMR, FTIR and melting temperature. All the monomer data were consistent with the published data¹¹.

Arg-Phe-PEAs were prepared according to the reaction scheme in Figure 7.4. The reaction conditions were intensively studied and optimized in terms of reaction time, reaction temperature, monomer concentrations, reaction medium and catalyst. The final optimized reaction conditions are: reaction temperature: 75 °C; concentration of each monomer: 1.0-1.5 mmol/mL; the reaction medium: DMA; catalyst (acid

acceptor): NEt_3 . All the Arg-Phe-PEAs were prepared with high yields ($> 80\%$) under the optimized reaction conditions. For the chemical structure identification of the prepared Arg-Phe-PEAs, the polymer chemical structure was confirmed by ^1H -NMR spectra. Figure 7.5 showed the ^1H -NMR spectrum of a representative Arg-Phe-PEA (8-Arg-6-S-8-Phe-4-20%). All the ^1H -NMR peaks of Arg-Phe-PEA were well identified, and the calculated Arg content is consistent with the theoretical value. The ^1H -NMR peaks marked with numbers from 1 to 20 are assigned to the corresponding protons as shown in Figure 7.5, which strongly supports the anticipated chemical structure of the Arg-Phe-PEA.

7.D.2 Solubility

The solubility of Arg-Phe-PEAs can greatly affect their potential applications. All Arg-Phe-PEAs synthesized in this study were water insoluble (Table 7.2). The Arg-Phe-PEAs copolymers with Arg contents ranging from 10 % to 20 % dissolved in DMSO and DMF, and showed some significant solubility difference from the pure Arg-PEA and Phe-PEA homopolymers. Compared with pure Arg-PEA, the Arg-Phe-PEAs copolymers were water insoluble; while compared with pure Phe-PEA, they were insoluble in THF, chloroform.

7.D.3 Static Contact Angle

The contact angle of Phe-PEA and Arg-Phe-PEA were measured and compared (Table 7.3). It was found that the Arg-Phe-PEAs showed significantly smaller contact angle values than the pure Phe-PEA homopolymer, i.e., 47 – 63% reduction due to the incorporation of Arg into Phe-PEAs. An increase in Arg content in the Arg-Phe-PEAs

(from 10% to 20%) reduced the contact angle further, i.e., more hydrophilic. The contact angle is the angle formed by a liquid at the three phase boundary where the liquid, gas, and solid intersect. The small contact angle means that the adhesive forces are dominating, while the high contact angle means the cohesive forces are dominating. So the introducing of Arg into Phe-PEAs brought significant changes (from hydrophobic to hydrophilic) to the Arg-Phe-PEA polymer surface property (contact angle) compared with pure Phe-PEA and more L-arginine percent in the polymer composition made the polymer more hydrophilic.

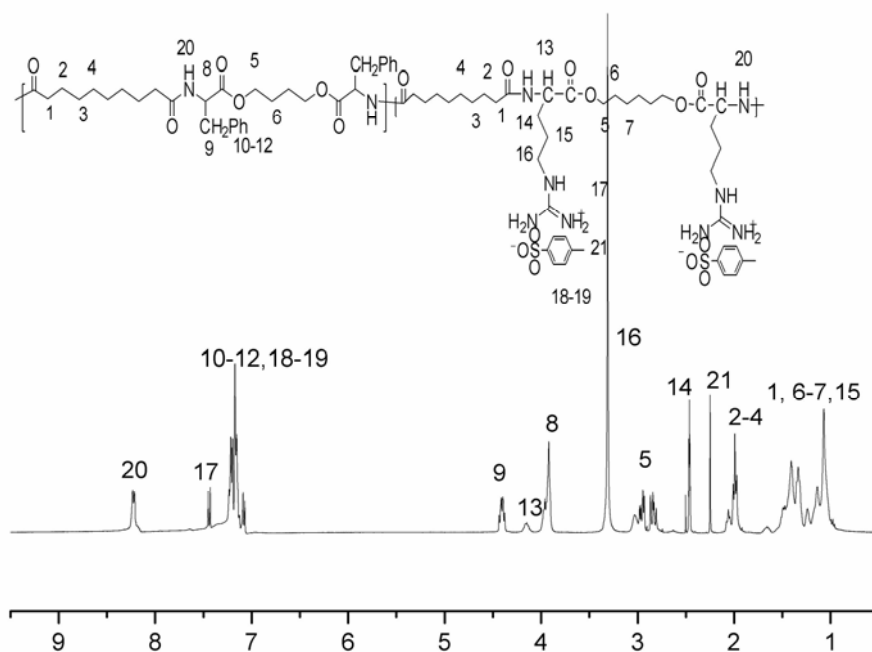


Figure 7.5 ¹H NMR spectrum of 8-Arg-6-S-8-Phe-4-20%

Table 7.2 Solubility of Arg-Phe-PEA

Arg-Phe- PEAs	Water	Ethanol	Acetone	THF	Chloroform	DMF	DMSO
4-Arg-4-S- 8-Phe-4- 10%	-	-	-	-	-	+	+
4-Arg-4-S- 8-Phe-4- 20%	-	-	-	-	-	+	+
8-Arg-6-S- 8-Phe-4- 10%	-	-	-	-	-	+	+
8-Arg-6-S- 8-Phe-4- 20%	-	-	-	-	-	+	+
8-Arg-6-S	+	-	-	-	-	+	+
4-Arg-4-S	+	-	-	-	-	+	+
8-Phe-4	-	-	-	+	+	+	+

* The solubility of polymers was measured at 25 °C and 1.0 mg/mL was used as a standard to determine whether a polymer is soluble or insoluble: + means soluble, - means insoluble

7.D.4 Thermal Property of Arg-Phe-PEAs

Table 7.4 and Figure 7.6 show how the glass transition temperature (T_g) of Arg-Phe-PEA copolymer is different from the pure Arg-PEA and Phe-PEA, and how the copolymer composition affected the T_g of copolymer. Figure 7.6 (plotted according to the Gordon-Taylor-Wood equation ($k=0.21$)^{31, 32}) showed how the T_g of Arg-Phe-PEA copolymers depended on the Arg-PEA weight fraction in the copolymers. The following is the modified Gordon-Taylor-Wood equation: $(T_g)_{\text{Arg-Phe-PEA}} = [W_{\text{Arg-PEA}} \cdot (T_g)_{\text{Arg-PEA}} + k \cdot W_{\text{Phe-PEA}} \cdot (T_g)_{\text{Phe-PEA}}] / [W_{\text{Arg-PEA}} + k \cdot W_{\text{Phe-PEA}}]$ ^{31, 32}. The data in Table 7.4 and Figure 7.6 demonstrate that the T_g value of Arg-Phe-PEA is affected by both of the Phe and Arg contents, and the T_g value of Arg-Phe-PEA is between the T_g values of pure Phe-PEA and pure Arg-PEA. Since pure Phe-PEA has a higher T_g value than that of a pure Arg-PEA, it's expected that an increase in the percentage of Arg component in the Arg-Phe-PEA would result in a lower T_g of the hybrid Arg-Phe-PEA as demonstrated in Table 7.4 and Figure 7.6. Although the percentage of Arg content in the Arg-Phe-PEA copolymer is small (10 % or 20 %), the reduction in T_g is obvious. From Table 7.4, it was found that the introduction of 10% Arg content caused about 3 ° reduction of T_g . From Figure 7.6, we could find that the trend of T_g change almost has a linear relationship with the percentage of 8-Arg-6-S contents of the copolymer. The reason could be due to the significant change of the free volume of the copolymer structure as the pendant guanidine group from the Arg could increase the free volume of the copolymer chain and hence lower its T_g . The PEA copolymer composition effect on T_g was also reported by Guo et al in their study of unsaturated and saturated Phe-based PEAs⁹. They observed the similar trend and relationship for the T_g dependence on the unsaturated to saturated blocks of Phe-PEA: the copolymer T_g value would be between the T_g value of a pure saturated and unsaturated Phe-based

PEAs. The main difference between Guo et al.'s system and our system is that Kai et al introduced an unsaturated component (C=C double bond) into the backbone of the Phe-based PEA⁹, while we used hydrophilic positively charged component (Arg block) to couple with the hydrophobic Phe block.

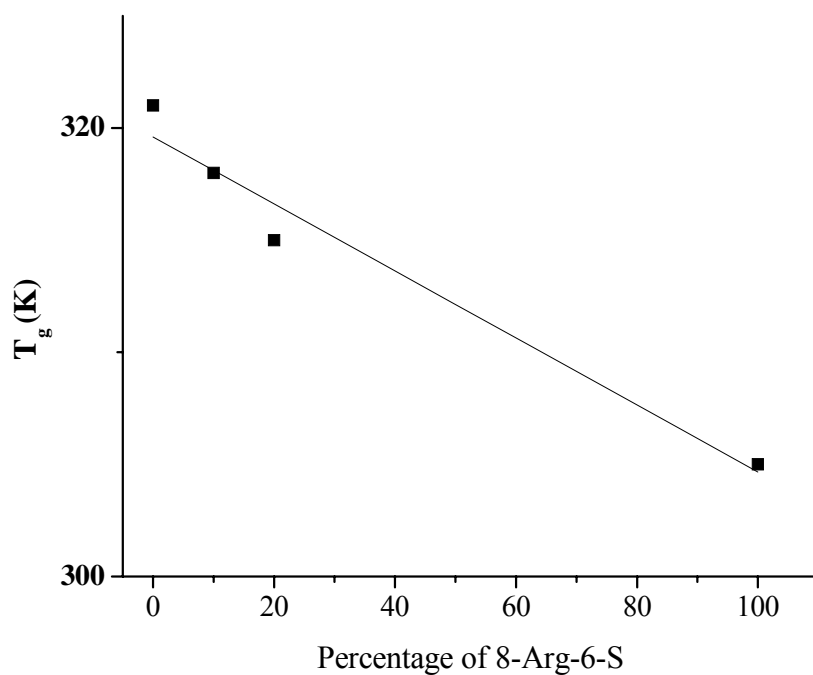


Figure 7.6 T_g of Arg-Phe-PEA copolymers as a function of the Arg-PEA weight fraction in the copolymers, plotted according to the Gordon-Taylor-Wood equation ($k=0.21$)^{31, 32}. T=329.6 K for x=0 and T=304.7 K for x=100, x is the percentage of 8-Arg-6-S.

7.D.5 In vitro Enzymatic Biodegradation of Arg-Phe-PEA

Three types of polymer films were selected for the enzymatic (α -chymotrypsin, 0.2 mg/mL) biodegradation tests. They are: 8-Phe-4, 8-Arg-4-S-8-Phe-4-10% and 8-Arg-4-S-8-Phe-4-20%. Figure 7.7 shows the enzymatic biodegradation results of the 3 polymers within 3 weeks period. All 3 PEA samples show near linear weight loss profiles, suggestion that they were biodegraded under a surface erosion mechanism. From Figure 7.7, 8-Phe-4 film showed the fastest biodegradation rate as it was completely degraded within 12 days; the two Arg-Phe-PEA films showed the similar degradation profile as the 8-Phe-4, but with slightly slow rates, and the Arg-Phe-PEAs having a higher Arg contents resulted in a slower biodegradation rate, e.g., 19 days for a complete weight loss of 8-Arg-4-S-8-Phe-4-20% vs. 15 days for 8-Arg-4-S-8-Phe-4-10% .

According to the previous publications^{7, 9-11, 18-20, 33, 34}, the Phe-PEA could be biodegraded by α -chymotrypsin within a few days to several weeks, and the degradation rate depends on the enzyme concentration and the type of PEAs and their formulation (powder, particle or solid film). Because α -chymotrypsin works for hydrophobic amino acid based polymers, the Arg-Phe-PEA copolymer having relatively more Phe contents (less Arg contents) would show a faster biodegradation rate. Thus, the degradation rate of this type of Arg-Phe-PEA copolymers could be controlled by adjusting the Arg-Phe-PEA copolymer composition.

The surface morphology changes of the Arg-Phe-PEA copolymer films upon biodegradation are shown in Figure 7.8. After 6 days of incubation, the 8-Arg-4-S-8-Phe-4-20%. film showed a significant enzymatic catalyzed biodegradation as evident

by the appearance of visible eroded surface with many pores. After 10 days, the films were broken into small fragments. Kai et al also reported the investigation of biodegradation of ethylene glycol based Phe-PEA^{9, 19}, the results showed that the enzyme degradation rate of PEAs is significantly affected by the PEA types. For example, within 24 hr, ethylene glycol based Phe-PEA had a weight loss for around 80 wt %, while unsaturated PEA and saturated diol based PEA (such as 8-Phe-4) showed a weight loss of around 14 % and 6 %, respectively. The biodegradation data of the saturated diol based PEA (8-Phe-4) was consistent with our results. When comparing the SEM images of the enzyme biodegraded Arg-Phe-PEA films with the published SEM images of Phe-PEAs^{9, 19}, similar surface morphology between these 2 polymer systems was observed.

Table 7.3 Static contact angle of polymers

Polymer Name	PCL	8-Phe-4	Arg-Phe-PEA-1 (10 % Arg)	Arg-Phe-PEA-2 (20 % Arg)
Static Angle(°)	90.86±0.77	79.62±1.03	42.51±0.91	29.17±1.47

Table 7.4 T_g value of Phe-PEA, Arg-PEA and Arg-Phe-PEA

PEA	8-Phe-4	8-Arg-6-S-8-Phe-4- 10%	8-Arg-6-S-8-Phe-4- 20%	8-Arg-6-S
T _g (°C)	48±2	45±2	42±2	32±2

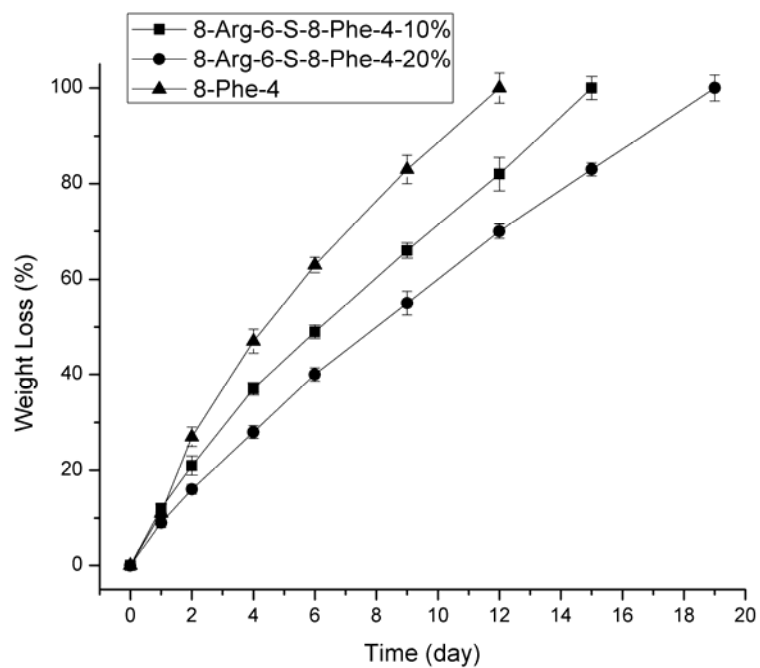


Figure 7.7 Enzymatic biodegradation of Arg-Phe-PEA in α -chymotrypsin (0.2 mg/mL) solution in terms of weight loss

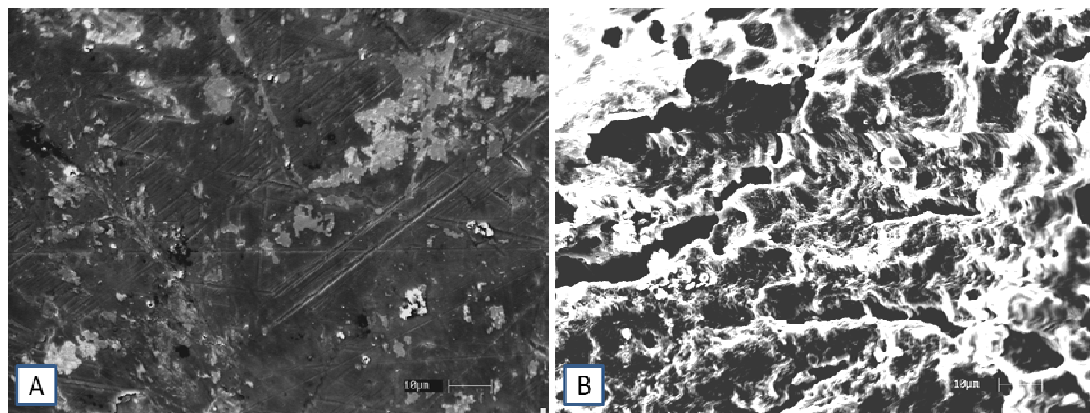


Figure 7.8 SEM images of 8-Arg-4-S-8-Phe-4-20% polymer film incubated in 0.20 mg/mL α -chymotrypsin solution at 37 °C: a, incubated for 0 days; b, incubated for 6 days.

7.D.6 Cell Attachment and Proliferation Assays

Four types of PEA-polymer coatings were tested for BAEC attachment and proliferation. They are: collagen, 8-Phe-4, PBMA, Arg-Phe-PEA (8-Arg-6-S-8-Phe-4-20%). The cell culture plate coated with collagen was used here as a control and PBMA coated glass slide was used as a polymer control. The cell morphology data (Figure 7.9, 96 h) indicate that the cultured BAEC showed very good attachment and proliferation behavior on collagen, 8-Phe-4 and Arg-Phe-PEA coatings, while the PBMA could not support cell proliferation as well. When compared with collagen and 8-Phe-4, the cell attachment performance on the Arg-Phe-PEA coating was almost at the same level.

For the proliferation tests, an increase in cell number (cell proliferation) results in an increase in the amounts of MTT formazan produced and an increase in UV absorbance (optical density). Figure 7.10 showed that the PBMA could not support the BAEC proliferation well, while collagen, Arg-Phe-PEA and 8-Phe-4 could support the cell proliferation pretty well, and the Arg-Phe-PEA copolymer showed almost the same cell proliferation level as the 8-Phe-4 and collagen. The Dunnet statistical data analysis showed that there was no significant difference between the Arg-PEA treatment and the collagen control (or 8-Phe-4) at the p value of 0.05 level. So there is no evidence of cytotoxicity of Arg-PEAs. Therefore, based on the data from Figures 7.9 and 7.10, the new positively charged water insoluble Arg-Phe-PEAs were nontoxic to the cells and could promote the cell attachment and proliferation, suggesting that the Arg-Phe-PEAs could be a good candidate for tissue engineering scaffold.

The excellent BAEC cell attachment and proliferation performance on Arg-Phe-PEA surface is consistent with the reported the cationic Lys-Phe-PEA³⁵. Horwitz et al reported how the surface charge property affected the BAEC cell attachment and proliferation³⁵. They investigated three types of PEAs of different charge property (positive, neutral and negative), but with similar surface hydrophobicity (contact angle). Their results indicated that negatively charged PEAs could not support the BAEC attachment and proliferation well, while positively charged and neutral PEAs showed excellent BAEC attachment and proliferation³⁵. When comparing the reported cationic Lys-Phe-PEA and the current cationic Arg-Phe-PEA, both have Phe-PEA component and positive charge, but with significant difference of the contact angle values (Lys-Phe-PEA is around 76°³⁵, while Arg-Phe-PEA is around 30-40°) indicating that the two types of polymers had very different hydrophobic/hydrophilic surface property. Thus, the similar cell attachment and proliferation performance

between the prior reported cationic Lys-Phe-PEA and the current cationic Arg-Phe-PEA suggests that the surface charge property maybe the dominant factor for cell attachment. It is reasonable that a positively charged surface could facilitate the deposition of proteins necessary for cell attachment.

7.D.7 In vitro Inflammatory Response of Arg-Phe-PEAs

In this study, the inflammatory responses to these new Arg-Phe-PEAs were assessed by examining the mouse macrophage activation after 48 hours incubation with polymer samples, and TNF- α released was measured by using an ELISA kit. PMBA was used as a polymer control because it's a widely used FDA-approved biomaterial in medical devices like drug-eluting stents. PCL was also used as a polymer control because it is a FDA approved absorbable biomaterial. The TNF- α release data in Figure 7.11 show that the positive control (LPS) showed a very high level inflammatory response as expected. PBMA and PCL showed medium level inflammation response, while Arg-Phe-PEA showed a significantly low level inflammation response, almost the same as the macrophage group ($M\Phi$) without any treatment. The very low inflammation response will bring Arg-Phe-PEA potential applications for the medical device area.

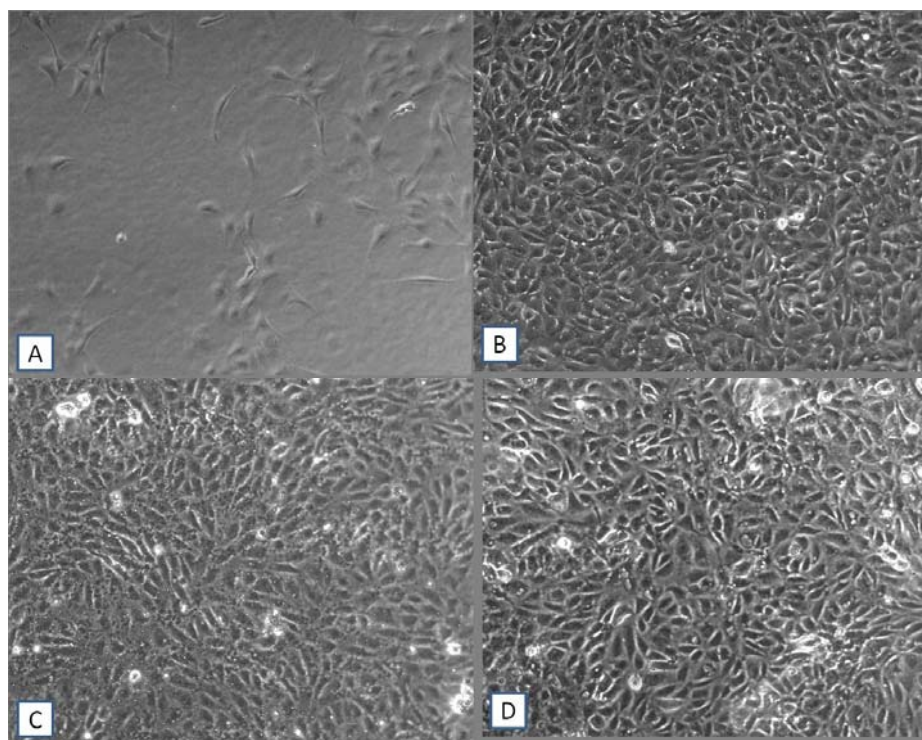


Figure 7.9 Microscopic image of the attached BAEC on the polymer coated glass coverslips. 10x, and 96h: (A) PBMA coated ;(B) collagen coated; (C) 8-Phe-4 coated; (D) 8-Arg-6-S-8-Phe-4-20% coated;

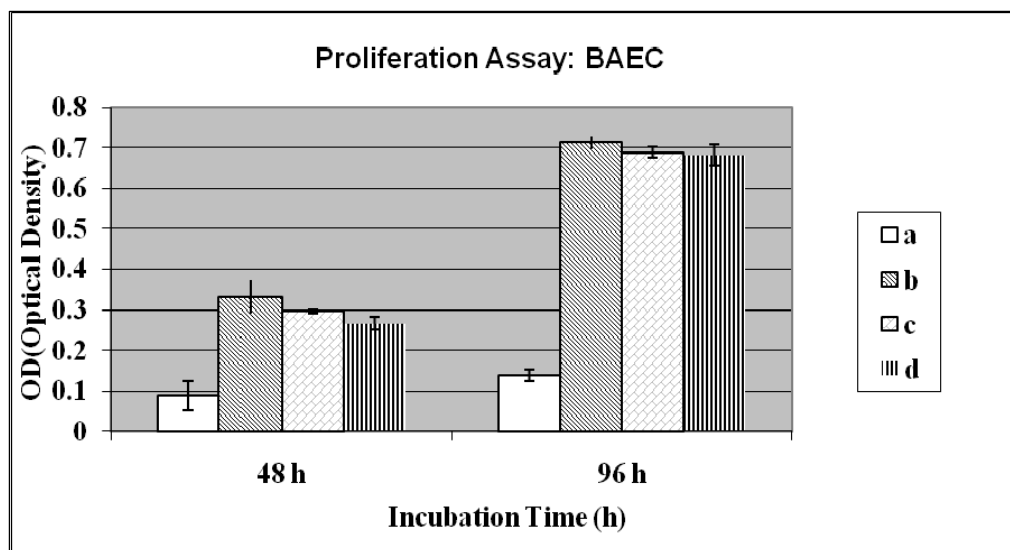


Figure 7.10 Proliferation assay of BAEC on polymer coatings on glass coverslips (a) PBMA coated; (b) collagen coated; (c) 8-Phe-4 coated; (d) 8-Arg-6-S-8-Phe-4-20% coated

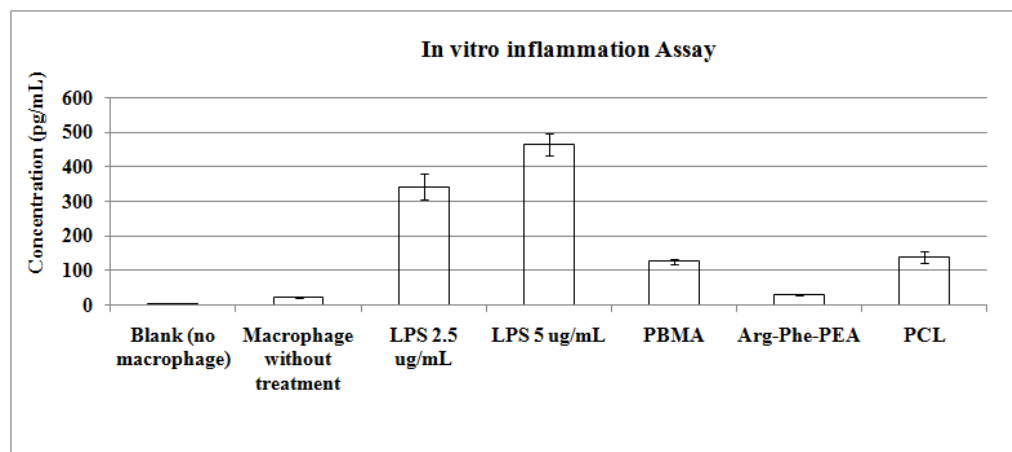


Figure 7.11 in vitro measurement of inflammatory response of polymers (Arg-Phe-PEA: 8-Arg-6-S-8-Phe-4-20%) in terms of concentration of TNF- α released from J774 macrophages

7.E Conclusion

A new family of water insoluble ionic and biodegradable hybrid poly (ester amide) copolymers (Arg-Phe-PEAs) has been successfully synthesized and characterized. The introduction of Arg moiety into Phe-PEAs could greatly alter the surface and bulk properties of Phe-PEAs, such as contact angle value and glass transition temperature. Enzyme biodegradation tests showed that the enzyme degradation rate of the Arg-Phe-PEAs was via surface-erosion mode and could be controlled by adjusting the Arg to Phe feed ratio during a solution polycondensation. *In vitro* cell attachment and proliferation tests showed that the new Arg-Phe-PEA copolymers could promote the BAEC cell attachment and proliferation as well as the pure Phe-PEA and collagen. The *in vitro* inflammatory response test indicated that the Arg-Phe-PEAs had very low inflammatory response when comparing with 2 commercial FDA approved polymers. Therefore, this new Arg-Phe-PEA family could expand the applications of PEAs and may be good potential candidates for tissue engineering scaffolds and drug delivery. The synthesis route of Arg-Phe-PEA could be easily applied to other amino acid based PEAs.

REFERENCE

1. Barrera, D. A.; Zylstra, E.; Lansbury, P. T., Jr.; Langer, R., Synthesis and RGD peptide modification of a new biodegradable copolymer: poly(lactic acid-co-lysine). *Journal of the American Chemical Society* **1993**, 115, (23), 11010-11.
2. Langer, R., Biomaterials in Drug Delivery and Tissue Engineering: One Laboratory's Experience. *Accounts of Chemical Research* **2000**, 33, (2), 94-101.
3. Langer, R.; Tirrell, D. A., Designing materials for biology and medicine. *Nature (London, United Kingdom)* **2004**, 428, (6982), 487-492.
4. Uhrich, K. E.; Cannizzaro, S. M.; Langer, R. S.; Shakesheff, K. M., Polymeric Systems for Controlled Drug Release. *Chemical Reviews (Washington, D. C.)* **1999**, 99, (11), 3181-3198.
5. Wang, Y.; Ameer, G. A.; Sheppard, B. J.; Langer, R., A tough biodegradable elastomer. *Nature Biotechnology* **2002**, 20, (6), 602-606.
6. West, J. L., Drug delivery: Pulsed polymers. *Nature Materials* **2003**, 2, (11), 709-710.
7. Chu, C.-C., Biodegradable polymeric biomaterials: an updated overview. *Biomaterials* **2007**, 6/1-6/22.
8. Wu, J.; Wang, X.; Keum, J. K.; Zhou, H.; Gelfer, M.; Avila-Orta, C.-A.; Pan, H.; Chen, W.; Chiao, S.-M.; Hsiao, B. S.; Chu, B., Water soluble complexes of chitosan-g-MPEG and hyaluronic acid. *Journal of Biomedical Materials Research, Part A* **2007**, 80A, (4), 800-812.
9. Guo, K.; Chu, C. C., Synthesis, characterization, and biodegradation of copolymers of unsaturated and saturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2007**, 45, (9), 1595-1606.

10. Guo, K.; Chu, C. C.; Chkhaidze, E.; Katsarava, R., Synthesis and characterization of novel biodegradable unsaturated poly(ester amide)s. *Journal of Polymer Science, Part A: Polymer Chemistry* **2005**, 43, (7), 1463-1477.
11. Katsarava, R.; Beridze, V.; Arabuli, N.; Kharadze, D.; Chu, C. C.; Won, C. Y., Amino acid-based bioanalogous polymers. synthesis, and study of regular poly(ester amide)s based on bis(alpha -amino acid) alpha ,w-alkylene diesters, and aliphatic dicarboxylic acids. *Journal of Polymer Science, Part A: Polymer Chemistry* **1999**, 37, (4), 391-407.
12. Deng, M.; Wu, J.; Reinhart-King, C. A.; Chu, C.-C., Synthesis and Characterization of Biodegradable Poly(ester amide)s with Pendant Amine Functional Groups and in Vitro Cellular Response. *Biomacromolecules* **2009**, 10, (11), 3037-3047.
13. Vera, M.; Franco, L.; Puiggali, J., Synthesis of poly(ester amide)s with lateral groups from a bulk polycondensation reaction with formation of sodium chloride salts. *Journal of Polymer Science, Part A: Polymer Chemistry* **2007**, 46, (2), 661-667.
14. Fan, Y.; Kobayashi, M.; Kise, H., Synthesis and biodegradation of poly(ester amide)s containing amino acid residues: the effect of the stereoisomeric composition of L- and D-phenylalanines on the enzymatic degradation of the polymers. *Journal of Polymer Science, Part A: Polymer Chemistry* **2001**, 40, (3), 385-392.
15. Yamanouchi, D.; Wu, J.; Lazar, A. N.; Craig Kent, K.; Chu, C.-C.; Liu, B., Biodegradable arginine-based poly(ester-amide)s as non-viral gene delivery reagents. *Biomaterials* **2008**, 29, (22), 3269-3277.
16. Chu, C.-C., Biodegradable polymeric biomaterials: an updated overview. *Biomaterials* **2003**, 95-115.
17. Guo, K.; Chu, C. C., Biodegradation of unsaturated poly(ester-amide)s and their hydrogels. *Biomaterials* **2007**, 28, (22), 3284-3294.

18. Guo, K.; Chu, C. C., Controlled release of paclitaxel from biodegradable unsaturated poly(ester amide)s/poly(ethylene glycol) diacrylate hydrogels. *Journal of Biomaterials Science, Polymer Edition* **2007**, 18, (5), 489-504.
19. Guo, K.; Chu, C. C., Copolymers of unsaturated and saturated poly(ether ester amide)s: synthesis, characterization, and biodegradation. *Journal of Applied Polymer Science* **2008**, 110, (3), 1858-1869.
20. Guo, K.; Chu, C. C., Biodegradable and injectable paclitaxel-loaded poly(ester amide)s micropheres: fabrication and characterization. *Journal of Biomedical Materials Research, Part B: Applied Biomaterials* **2009**, 89B, (2), 491-500.
21. Huang, S. J.; Bansleben, D. A.; Knox, J. R., Biodegradable polymers: Chymotrypsin degradation of a low molecular weight poly(ester-urea) containing phenylalanine. *Journal of Applied Polymer Science* **1979**, 23, (2), 429-37.
22. Song, H. L-arginine based biodegradable poly(ester amide)s, their synthesis, characterization, fabrications and applications as drug and gene carriers. 2007.
23. Li, L.; Chu, C.-C., Nitroxyl radical incorporated electrospun biodegradable poly(ester amide) nanofiber membranes. *Journal of Biomaterials Science, Polymer Edition* **2009**, 20, (3), 341-361.
24. Pang, X.; Chu, C.-C., Synthesis, characterization and biodegradation of functionalized amino acid-based poly(ester amide)s. *Biomaterials* **2010**, 31, (14), 3745-3754.
25. Guo, K.; Chu, C. C., Synthesis of biodegradable amino-acid-based poly(ester amide)s and poly(ether ester amide)s with pendant functional groups. *Journal of Applied Polymer Science* **2010**, 117, (6), 3386-3394.
26. Vera, M.; Almontassir, A.; Rodriguez-Galan, A.; Puiggali, J., Synthesis and Characterization of a New Degradable Poly(ester amide) Derived from 6-Amino-1-hexanol and Glutaric Acid. *Macromolecules* **2003**, 36, (26), 9784-9796.

27. Jokhadze, G.; Machaidze, M.; Panosyan, H.; Chu, C. C.; Katsarava, R., Synthesis and characterization of functional elastomeric poly(ester amide) copolymers. *Journal of Biomaterials Science -- Polymer Edition* **2007**, 18, (4), 411-438.
28. Pang, X.; Wu, J.; Reinhart-King, C.; Chu, C.-C., Synthesis and characterization of functionalized water soluble cationic poly(ester amide)s. *Journal of Polymer Science, Part A Polymer Chemistry* **2010**, 48, (17), 3758-3766.
29. De Wit, M. A.; Wang, Z.; Atkins, K. M.; Mequanint, K.; Gillies, E. R., Syntheses, characterization, and functionalization of poly(ester amide)s with pendant amine functional groups. *Journal of Polymer Science, Part A Polymer Chemistry* **2008**, 46, (19), 6376-6392.
30. Bhatia, S. K.; Arthur, S. D., Poly(vinyl alcohol) acetoacetate-based tissue adhesives are non-cytotoxic and non-inflammatory. *Biotechnol. Lett.* **2008**, 30, (8), 1339-1345.
31. Gordon, M.; Taylor, J. S., Ideal copolymers and the second-order transitions of synthetic rubbers. I. Noncrystalline copolymers. *J. Appl. Chem.* **1952**, 2, 493-500.
32. Wood, L. A., Glass transition temperatures of copolymers. *J. Polym. Sci.* **1958**, 28, 319-30.
33. Chu, C. C., Biodegradable hydrogels as drug controlled release vehicles. *Tissue Engineering and Novel Delivery Systems* **2004**, 423-461.
34. Tsitlanadze, G.; Machaidze, M.; Kviria, T.; Djavakhishvili, N.; Chu, C. C.; Katsarava, R., Biodegradation of amino-acid-based poly(ester amide)s: In vitro weight loss and preliminary in vivo studies. *Journal of Biomaterials Science, Polymer Edition* **2004**, 15, (1), 1-24.
35. Horwitz, J. A.; Shum, K. M.; Bodle, J. C.; Deng, M.; Chu, C.-C.; Reinhart-King, C. A., Biological performance of biodegradable amino acid-based poly(ester

amide)s: endothelial cell adhesion and inflammation in vitro. *J. Biomed. Mater. Res., Part A* **2010**, 95A, (2), 371-380.

APPENDICES

Appendix 1: Chapter 3 Water Soluble Arginine Poly (ester amide) as Gene Delivery Vector and Investigation of Structure-Function Relationship

A1.1 Transfection and cytotoxicity evaluation of SMC A10 and RSMC primary cell

A1.1.1 MTT assay for cytotoxicity evaluation

SMC A10 and RSMC primary cells were obtained from American Tissue Culture Collection (ATCC). The cells were grown as recommended at 37 °C in 5 % CO₂ in Dulbecco's minimal essential medium (DMEM) supplemented with 10 % FBS (Germini, Woodland, CA) and 1 % penicillin–streptomycin solution. The evaluation of the cytotoxicity of Arg-PEA/DNA complexes was performed by MTT assay. Cultured cells were seeded at an appropriate cell density concentration (10,000 cells/well) in 96-well plates and incubated overnight. Then the cells were treated with the freshly prepared aqueous Arg-PEA/DNA solutions. Cells without material treated were used as blank control. Cells treated with Superfect[®]/DNA, Lipofectamine2000[®]/DNA, PEI/DNA and PLL/DNA were used for comparison. After 48 h incubation, 20 µL of MTT solution (5 mg/mL) was added to each well, followed by 4 h incubation at 37 °C, 5 % CO₂. The cell culture medium was then carefully removed and 200 µL of acidic isopropyl alcohol (with 0.1 M HCl) was added to dissolve the formed formazan crystal. The plate was slightly shaken for 20 mins to make sure the crystal dissolved completely. Absorbance (OD) was measured immediately at 570 nm (subtract background reading at 690 nm) using a microplate reader (VersaMax Tunable Microplate reader Molecular Devices, USA). The cell viability (%) was calculated according to the following equation:

$$\text{Viability (\%)} = (\text{OD}_{570(\text{sample})} - \text{OD}_{620(\text{sample})}) / (\text{OD}_{570(\text{control})} - \text{OD}_{620(\text{control})}) \times 100\%$$

Where the $OD_{570(\text{control})}$ represents the measurement from the wells treated with medium only and the $OD_{570(\text{sample})}$ from the wells treated with various polymer/plasmid DNA complexes. (Error bars represent mean + SEM, * $P < 0.05$, number means the polymer/DNA weight ratio) As shown in Figure 1, the Arg-PEA treated groups showed comparable cell viabilities to the blank control group, while the Superfect[®], Lipofectamine2000[®], PEI and PLL treated groups showed significant reduction of cell viabilities. The Lipofectamine2000[®] and Superfect[®] were used as the recommendation of manufacture protocol. The DNA (μg): Lipofectamine2000[®] (μL) ratio was fixed at 1:2.5.

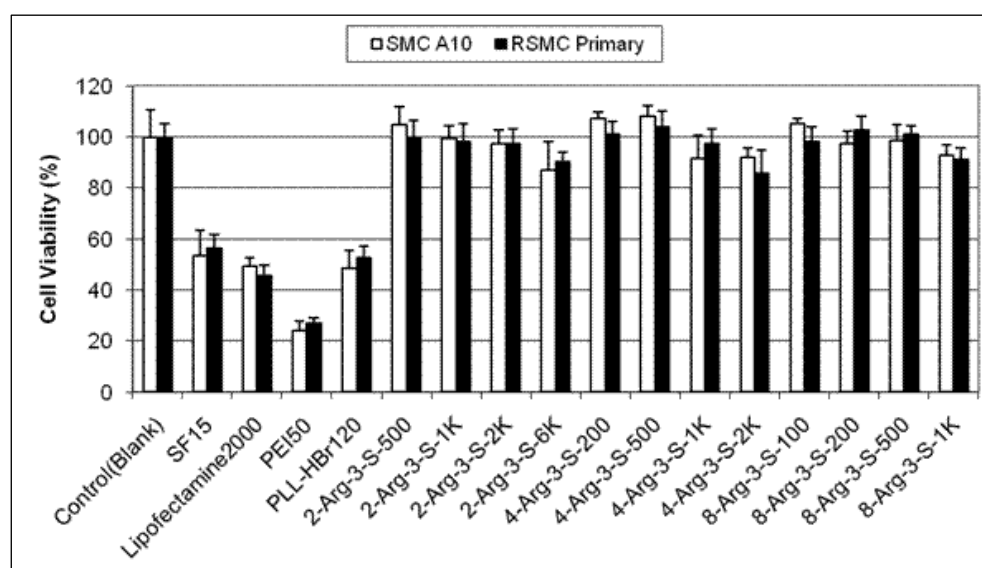


Figure 1 Cytotoxicity evaluation of Arg-PEA/DNA complexes by MTT assay. Control (blank) is the cells only without any material treatment. Various WRs of Arg-PEA to DNA were tested for 3 types of Arg-PEAs, from 2-Arg-3-S to 8-Arg-3-S. The numbers after the Arg-PEA, PEI, PLL and SF (Superfect[®]) name indicated the corresponding WR. Lipofectamine2000[®] and Superfect[®] were used as the recommendation of manufacture protocol.

A1.1.2 Firefly luciferase assay for transfection efficiency evaluation

Plasmid DNA was prepared and purified following the manufacturer's protocol. Arg-PEA 1X PBS buffer solution was freshly prepared in 1.5 mL microfuge tube right before the transfection test (start the transfection tests within 4 hours after making the Arg-PEA solution). For 2-Arg-3-S, the suggested solution concentration is 50 mg/mL. For 8-Arg-3-S, the suggested solution concentration is 10 mg/mL. If needed, slightly heat the solution in 60 degree isotherm heat bath to make sure all the polymers were completely dissolved. After that, vortex the solution for 5 seconds and let the polymer solution cool down to room temperature before making the Arg-PEA/DNA complex.

The Arg-PEA/ DNA complexes were prepared by adding the plasmid DNA solution with calculated volume to the freshly prepared Arg-PEA solutions to obtain systems with given DNA amount (1 μg /well for 24-well plate and 0.25 μg /well for 96-well plate) and weight ratios of Arg-PEA/DNA (for 2-Arg-3-S, the optimized ratio is 1000-3000). 2-3 weight ratios are suggested for each polymer. The following are some details for the complex preparation: add the calculated amount of DNA solution to polymer solution, then perform immediate pipe up and down for 3-5 seconds after mixing the solutions (do not vortex or centrifuge), and then equilibrate solution in the cell culture hood for 20-30 minutes at room temperature (UV irradiation is suggested).

The cells were seeded at 30×10^3 /well in a 24-well plate or 5×10^3 /well in a 96-well plate 24-48 hours before transfection (70 % confluent at transfection). For 24-well plate, 1 μg COL (-772)/LUC or GFP DNA was formulated with the different Arg-PEA solutions at various weight ratios. For Superfect[®] control, 1 μg of plasmid DNA in 60 μL serum-free DMEM were supplemented with 5 μL (3 μg / μL) of the

Superfect[®] (SF) solution according to manufacturer's recommendation. For Lipofectamine2000[®] control, 1 µg of plasmid DNA in 50 µL serum-free DMEM were supplemented with 2.5 µL of the Lipofectamine2000[®] solution in 50 µL serum-free DMEM according to manufacturer's recommendation. Immediately before transfection, cells were washed twice with 1X PBS, and then the 1 mL serum-free DMEM were added to each well. Arg-PEA/DNA complex solution and control solutions were then added into each well immediately, piped up and down for 2-3 seconds. Cells were transfected for 3-4 h (cell line) or 12-16 h (primary cells) at 37 °C (5 % CO₂), and then the media was removed. After that, 0.5 mL of complete media for cell (follow the ATCC protocol) were added to each well and kept incubation. Cells were harvested for luciferase reading or GFP reading after 48 hours. Luciferase assay was performed according to Promega's recommendation. Briefly, cells from each well of a 24-well plate were lysed in 100 µL lysis buffer, transferred to a micro-tube, and then centrifuged at 10 000 g for 2 min. Supernatants were collected and analyzed for luciferase activity. In a typical experiment 20 µL of supernatant was added to luminometric tubes containing 100 µL of luciferase substrate (Promega). Light emission was measured with a Dual-luciferase detection system for a period of 5 sec. the relative light units/s was determined. Triplicates were used in each experiment.

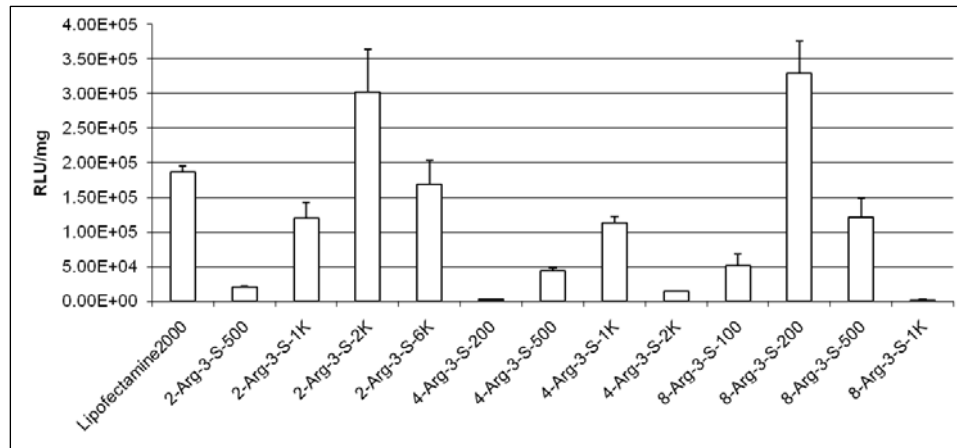


Figure 2 SMC A10 transfection efficiency of Arg-PEA/DNA complexes was expressed by firefly luciferase activity. Plasmid DNA used here was COL (-772)/Luc. Lipofectamine2000[®] was used as the recommendations of Invitrogen[®] protocol. Various WRs of Arg-PEA to DNA were tested and the number after the Arg-PEA was the corresponding WR.

A1.1.3 GFP assay

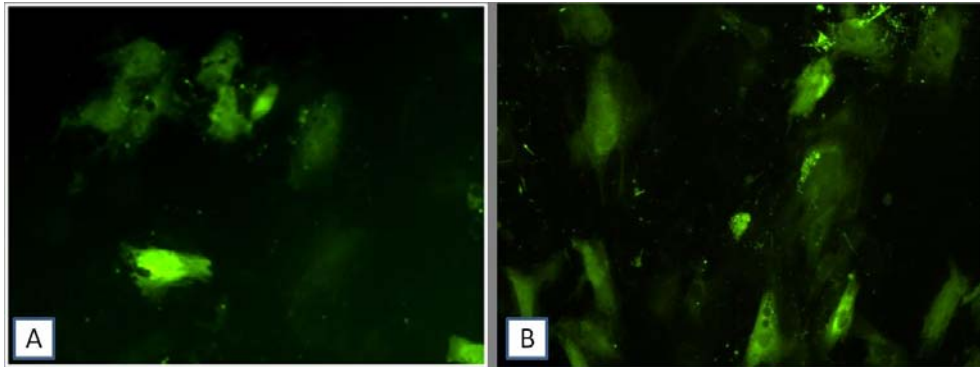


Figure 3 GFP transfection efficiency of SMC A10 was recorded under fluorescence microscope (10X). SMC A10 cells were transfected by (A) Lipofectamine2000[®] and (B) Arg-PEA (2-Arg-3-S, WR=2,000) (4h treatment and GFP images were taken after 48h). Lipofectamine2000[®] was used as the recommendations of Invitrogen[®] protocol

A1.1.4 Cell morphology (48 h, 10X) for RSMC primary cells

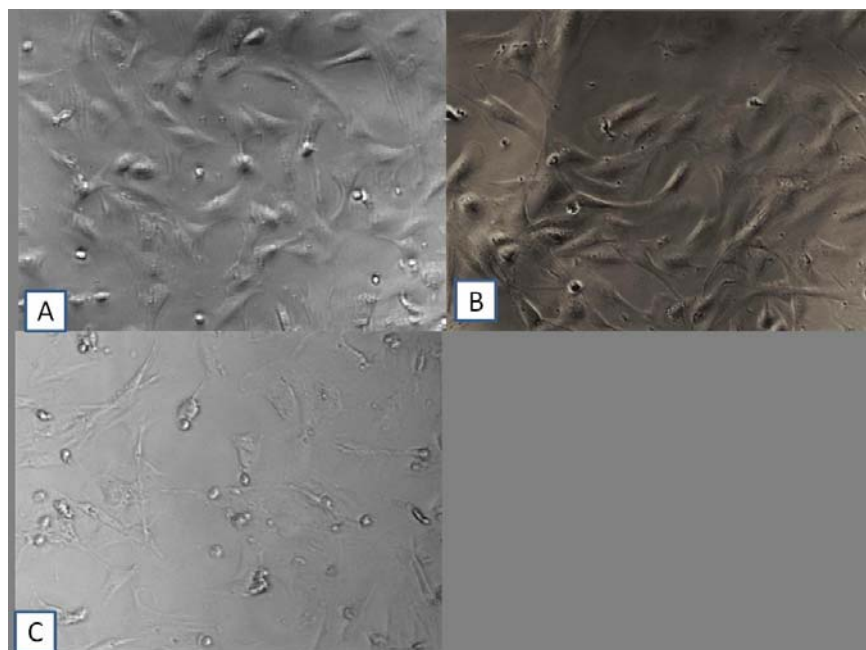


Figure 4 RSMC primary cell morphology was recorded under microscope (10X, 12 h treatment, pictures taken after 48h): (A) negative control, no material added; (B) cells treated with 2,000 µg 2-Arg-3-S/1 µg DNA per well (24 well); (C) cells treated with 3.0 µL Lipofectamine2000®/1 µg DNA per well (24 well).

A1.2 DNA delivery by UArg-PEA

A1.2.1 Firefly luciferase assay for transfection efficiency evaluation of UArg-PEA

Plasmid DNA was prepared and purified following the manufacturer's protocol. UArg-PEA 1X PBS buffer solution was freshly prepared in 1.5 mL microfuge tube right before the transfection test (start the transfection tests within 4 hours after

making the UArg-PEA solution). For 2-UArg-2-S, the suggested solution concentration is 10 mg/mL. For 2-UArg-4-S, the suggested solution concentration is 2 mg/mL. If needed, slightly heat the solution in 60 degree isotherm heat bath to make sure all the polymers were completely dissolved. After that, vortex the solution for 5 seconds and let the polymer solution cool down to room temperature before making the UArg-PEA/DNA complex. The UArg-PEA/ DNA complexes were prepared by adding the plasmid DNA solution with calculated volume to the freshly prepared UArg-PEA solutions to obtain systems with given DNA amount (1 μ g/well for 24-well plate and 0.25 μ g/well for 96-well plate) and weight ratios of UArg-PEA/DNA (for 2-uArg-2-S, the optimized ratio is 500-2000; for 2-UArg-4-S, the ratio is 200-500). 2-5 weight ratios are suggested for each polymer. The following are some details for the complex preparation: add the calculated amount of DNA solution to polymer solution, then perform immediate pipe up and down for 3-5 seconds after mixing the solutions (do not vortex or centrifuge), and then equilibrate solution in the cell culture hood for 20-30 minutes at room temperature (UV irradiation is suggested).

The cells were seeded at 30×10^3 /well in a 24-well plate or 5×10^3 /well in a 96-well plate 24-48 hours before transfection (70 % confluent at transfection). For 24-well plate, 1 μ g COL (-772)/LUC or GFP DNA was formulated with the different Arg-PEA solutions at various weight ratios. For Superfect[®] control, 1 μ g of plasmid DNA in 60 μ L serum-free DMEM were supplemented with 5 μ L (3 μ g/ μ L) of the Superfect[®] (SF) solution according to manufacturer's recommendation. For Lipofectamine2000[®] control, 1 μ g of plasmid DNA in 50 μ L serum-free DMEM were supplemented with 2.5 μ L of the Lipofectamine2000[®] solution in 50 μ L serum-free DMEM according to manufacturer's recommendation. Immediately before transfection, cells were washed twice with 1X PBS, and then the 1 mL serum-free

DMEM were added to each well. UArg-PEA/DNA complex solution and control solutions were then added into each well immediately, piped up and down for 2-3 seconds. Cells were transfected for 3-4 h cell line at 37 °C (5 % CO₂), and then the media was removed. After that, 0.5 mL of complete media for cell (follow the ATCC protocol) were added to each well and kept incubation. Cells were harvested for luciferase reading after 48 hours. Luciferase assay was performed according to Promega's recommendation. Briefly, cells from each well of a 24-well plate were lysed in 100 µL lysis buffer, transferred to a micro-tube, and then centrifuged at 10 000 g for 2 min. Supernatants were collected and analyzed for luciferase activity. In a typical experiment 20 µL of supernatant was added to luminometric tubes containing 100 µL of luciferase substrate (Promega). Light emission was measured with a Dual-luciferase detection system for a period of 5 sec. the relative light units/s was determined. Triplicates were used in each experiment.

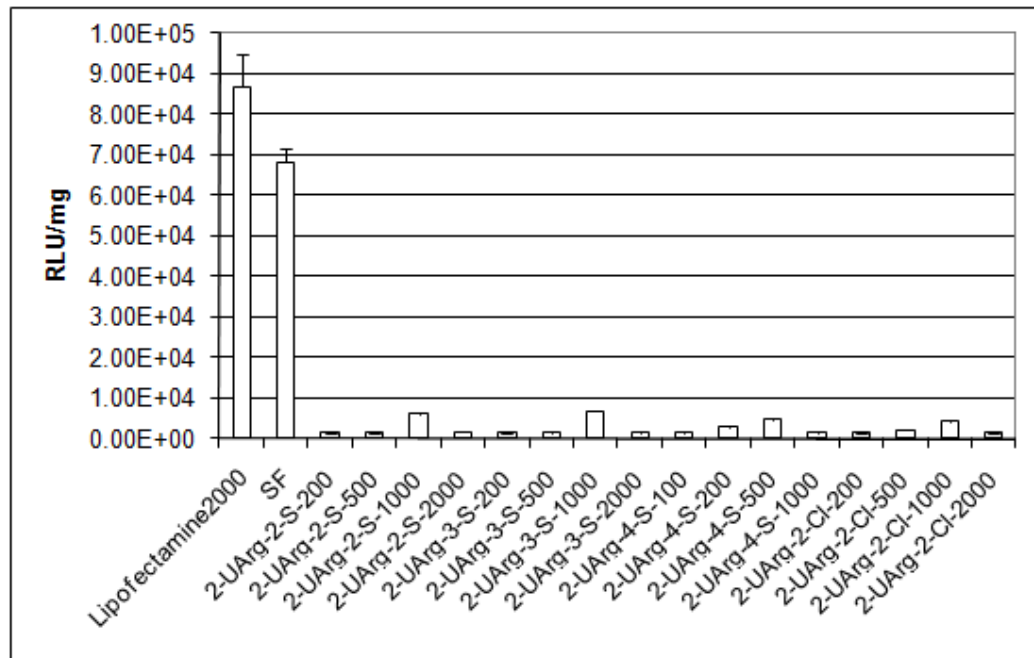


Figure 5 SMC A10 transfection efficiency of UArg-PEA/DNA complexes was expressed by firefly luciferase activity. Plasmid DNA used here was COL (-772)/Luc. Superfect[®] and Lipofectamine2000[®] were used as the recommendations of manufacturer protocol. Various WRs of Arg-PEA to DNA were tested and the number after the UArg-PEA was the corresponding WR.

Appendix 2: Chapter 4 Advanced Generation of Amino Acid-based Poly (ester amide)s as Non-viral Gene Delivery Vector for Primary and Stem Cells and Structure-Function Study

A2.1 Firefly luciferase assay for transfection efficiency evaluation of A10 SMC cell lines

Plasmid DNA was prepared and purified following the manufacturer's protocol. Arg-PEA 1X PBS buffer solution was freshly prepared in 1.5 mL microfuge tube right before the transfection test (start the transfection tests within 4 hours after making the Arg-PEA solution). For 8-Arg-3-S, the suggested solution concentration is 10 mg/mL. And for 2-Arg-6E-Cl (in the viscous state), the suggested concentration is 10 mg/mL. If needed, slightly heat the solution in 60 degree isotherm heat bath to make sure all the polymers were completely dissolved. After that, vortex the solution for 5 seconds and let the polymer solution cool down to room temperature before making the Arg-PEA/DNA complex.

The Arg-PEA/ DNA complexes were prepared by adding the plasmid DNA solution with calculated volume to the freshly prepared Arg-PEA solutions to obtain systems with given DNA amount (1 μ g/well for 24-well plate and 0.25 μ g/well for 96-well plate) and weight ratios of Arg-PEA/DNA (for 2-Arg-6E-CL, the ratio is 500-1000). 2-3 weight ratios are suggested for each polymer. The following are some details for the complex preparation: add the calculated amount of DNA solution to polymer solution, then perform immediate pipe up and down for 3-5 seconds after mixing the solutions (do not vortex or centrifuge), and then equilibrate solution in the cell culture hood for 20-30 minutes at room temperature (UV irradiation is suggested).

The cells were seeded at 30×10^3 /well in a 24-well plate or 5×10^3 /well in a 96-well plate 24-48 hours before transfection (70 % confluent at transfection). For 24-well plate, 1 μ g COL (-772)/LUC or GFP DNA was formulated with the different Arg-PEA solutions at various weight ratios. For Superfect[®] control, 1 μ g of plasmid DNA in 60 μ L serum-free DMEM were supplemented with 5 μ L (3 μ g/ μ L) of the Superfect[®] (SF) solution according to manufacturer's recommendation. For Lipofectamine2000[®] control, 1 μ g of plasmid DNA in 50 μ L serum-free DMEM were supplemented with 2.5 μ L of the Lipofectamine2000[®] solution in 50 μ L serum-free DMEM according to manufacturer's recommendation. Immediately before transfection, cells were washed twice with 1X PBS, and then the 1 mL serum-free DMEM were added to each well. Arg-PEA/DNA complex solution and control solutions were then added into each well immediately, pipe up and down for 2-3 seconds. Cells were transfected for 3-4 h (cells) at 37 °C (5 % CO₂), and then the media was removed. After that, 0.5 mL of complete media for cell (follow the ATCC protocol) were added to each well and kept incubation. Cells were harvested for luciferase reading or GFP reading after 48 hours. Luciferase assay was performed according to Promega's recommendation. Briefly, cells from each well of a 24-well plate were lysed in 100 μ L lysis buffer, transferred to a micro-tube, and then centrifuged at 10 000 g for 2 min. Supernatants were collected and analyzed for luciferase activity. In a typical experiment 20 μ L of supernatant was added to luminometric tubes containing 100 μ L of luciferase substrate (Promega). Light emission was measured with a Dual-luciferase detection system for a period of 5 sec. the relative light units/s was determined. Triplicates were used in each experiment.

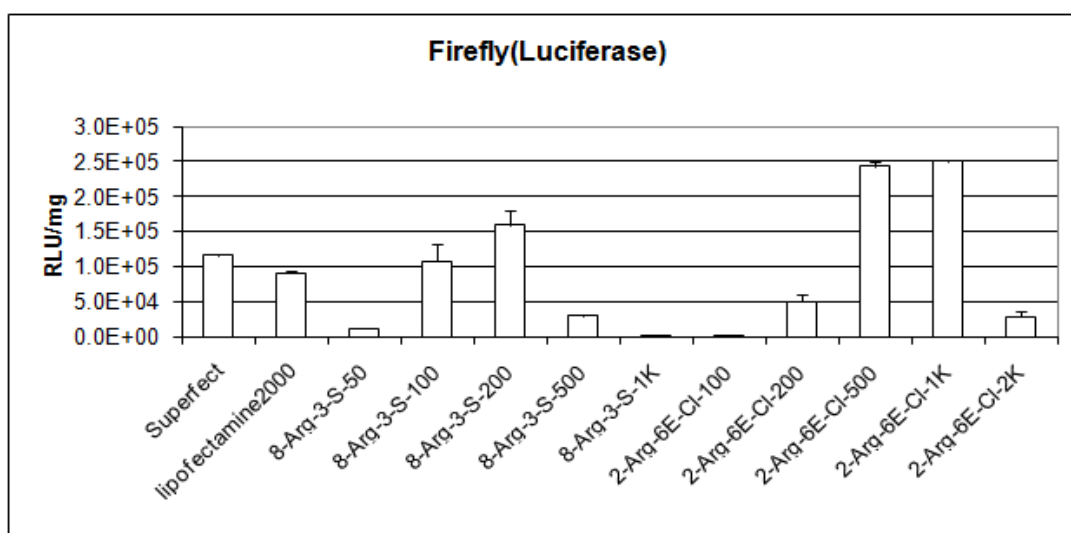


Figure 6 SMC A10 transfection efficiency of Arg-PEA/DNA complexes was expressed by firefly luciferase activity. Plasmid DNA used was COL (-772)/Luc. Lipofectamine2000[®] was used as the recommendations of Invitrogen[®] protocol. Various WRs of Arg-PEA to DNA were tested and the number after the Arg-PEAs was the corresponding WR.

A2.2 Transfection and cytotoxicity evaluation of SVEC4-10 endothelial cell

A2.2.1 Firefly luciferase assay for transfection efficiency evaluation

Plasmid DNA was prepared and purified following the manufacturer's protocol. Arg-PEA 1X PBS buffer solution was freshly prepared in 1.5 mL microfuge tube right before the transfection test (start the transfection tests within 4 hours after making the Arg-PEA solution). For 2-Arg-3-S, the suggested solution concentration is 50 mg/mL. For 8-Arg-3-S, the suggested solution concentration is 10 mg/mL. And for 2-Arg-6E-Cl (in the viscous state), the suggested concentration is 10 mg/mL. If needed, slightly heat the solution in 60 degree isotherm heat bath to make sure all the polymers were

completely dissolved. After that, vortex the solution for 5 seconds and let the polymer solution cool down to room temperature before making the Arg-PEA/DNA complex.

The Arg-PEA/ DNA complexes were prepared by adding the plasmid DNA solution with calculated volume to the freshly prepared Arg-PEA solutions to obtain systems with given DNA amount (1 $\mu\text{g}/\text{well}$ for 24-well plate and 0.25 $\mu\text{g}/\text{well}$ for 96-well plate) and weight ratios of Arg-PEA/DNA (for 2-Arg-3-S, the optimized ratio is 1000-3000; for 2-Arg-6E-CL, the ratio is 500-1000). 2-3 weight ratios are suggested for each polymer. The following are some details for the complex preparation: add the calculated amount of DNA solution to polymer solution, then perform immediate pipe up and down for 3-5 seconds after mixing the solutions (do not vortex or centrifuge), and then equilibrate solution in the cell culture hood for 20-30 minutes at room temperature (UV irradiation is suggested).

The cells were seeded at 30×10^3 /well in a 24-well plate or 5×10^3 /well in a 96-well plate 24-48 hours before transfection (70 % confluent at transfection). For 24-well plate, 1 μg COL (-772)/LUC or GFP DNA was formulated with the different Arg-PEA solutions at various weight ratios. For Superfect[®] control, 1 μg of plasmid DNA in 60 μL serum-free DMEM were supplemented with 5 μL (3 $\mu\text{g}/\mu\text{L}$) of the Superfect[®] (SF) solution according to manufacturer's recommendation. For Lipofectamine2000[®] control, 1 μg of plasmid DNA in 50 μL serum-free DMEM were supplemented with 2.5 μL of the Lipofectamine2000[®] solution in 50 μL serum-free DMEM according to manufacturer's recommendation. Immediately before transfection, cells were washed twice with 1X PBS, and then the 1 mL serum-free DMEM were added to each well. Arg-PEA/DNA complex solution and control solutions were then added into each well immediately, pipe up and down for 2-3

seconds. Cells were transfected for 3-4 h at 37 °C (5 % CO₂), and then the media was removed. After that, 0.5 mL of complete media for cell (follow the ATCC protocol) were added to each well and kept incubation. Cells were harvested for luciferase reading or GFP reading after 48 hours. Luciferase assay was performed according to Promega's recommendation. Briefly, cells from each well of a 24-well plate were lysed in 100 µL lysis buffer, transferred to a micro-tube, and then centrifuged at 10 000 g for 2 min. Supernatants were collected and analyzed for luciferase activity. In a typical experiment 20 µL of supernatant was added to luminometric tubes containing 100 µL of luciferase substrate (Promega). Light emission was measured with a Dual-luciferase detection system for a period of 5 sec. the relative light units/s was determined. Triplicates were used in each experiment.

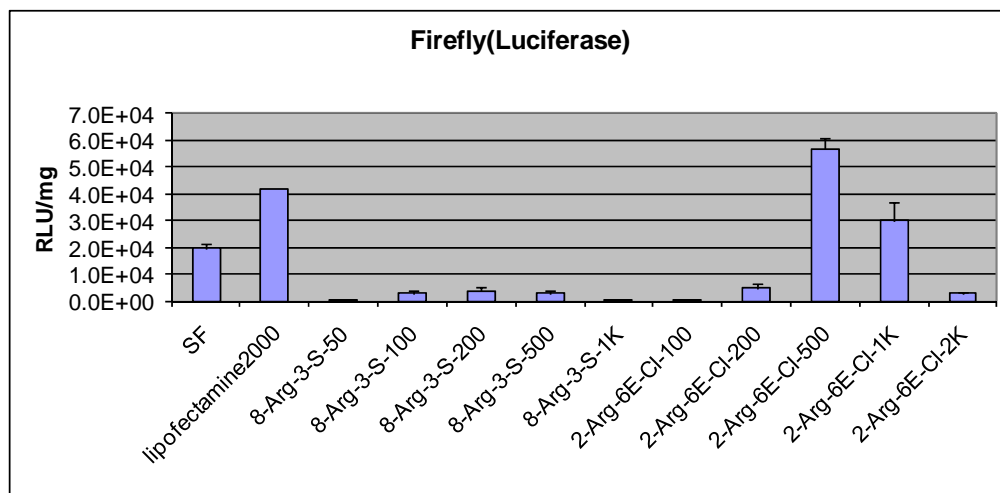


Figure 7 *SVEC4-10* endothelial cells transfection efficiency of Arg-PEA/DNA complexes was expressed by firefly luciferase activity. Plasmid DNA used was COL (-772)/Luc. Lipofectamine2000[®] and Superfect[®] (SF) was used as the recommendations of manufacture protocol. Various WRs of Arg-PEA to DNA were tested and the number after the Arg-PEAs was the corresponding WR.

A2.2.2 GFP assay

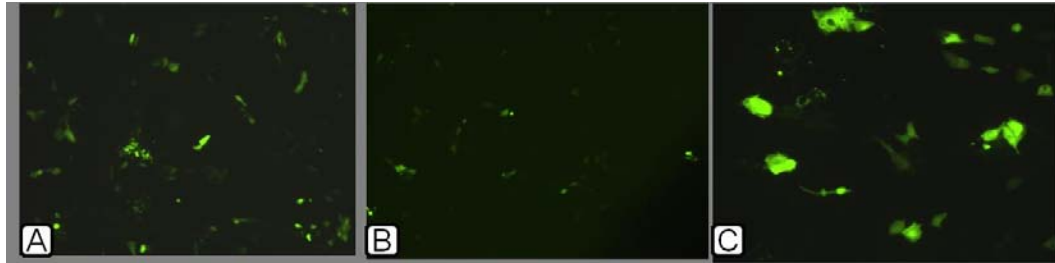


Figure 8 GFP transfection efficiency of *SVEC4-10* endothelial cells was recorded under fluorescence microscope (10X). *SVEC4-10* endothelial cells were transfected by (A) Lipofectamine2000[®], (B) Superfect[®], (C) 2-Arg-6E-Cl-1000(4h treatment and GFP images were taken after 48h). Lipofectamine2000[®] and Superfect[®] were used as the recommendations of manufacture protocol.

A2.2.3 MTT assay

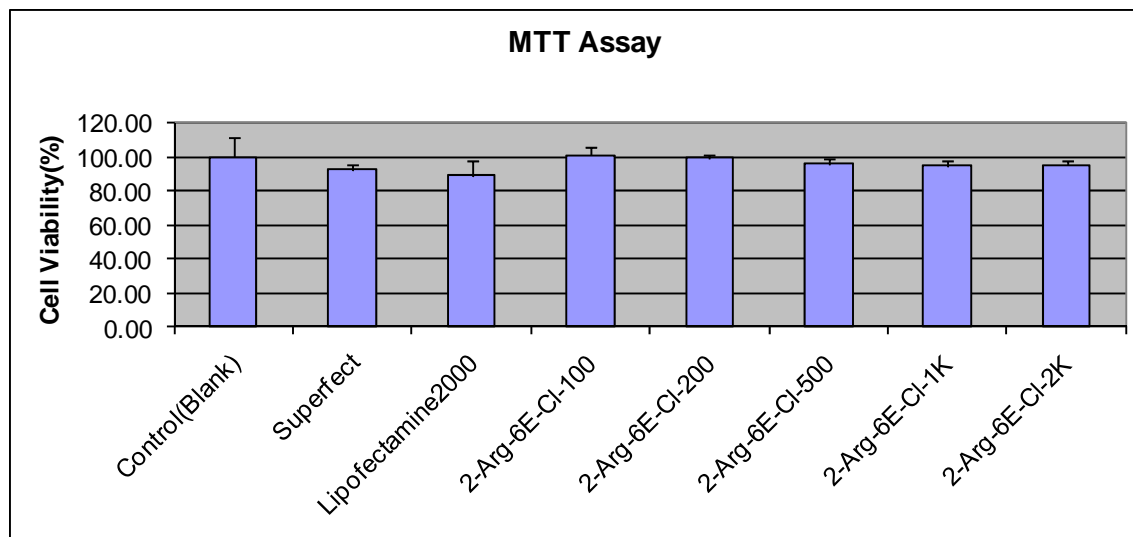


Figure 9 Cytotoxicity evaluation of Arg-PEEA/DNA complexes by MTT assay for *SVEC4-10* endothelial cells. Control (blank) is the cells only without any material treatment. Lipofectamine2000[®] and Superfect[®] were used as the recommendation of manufacture protocol.

A2.3 Transfection and cytotoxicity evaluation of RAW 264.7 macrophages

A2.3.1 Firefly luciferase assay for transfection efficiency evaluation

Plasmid DNA was prepared and purified following the manufacturer's protocol. Arg-PEA 1X PBS buffer solution was freshly prepared in 1.5 mL microfuge tube right before the transfection test (start the transfection tests within 4 hours after making the Arg-PEA solution). For 2-Arg-3-S, the suggested solution concentration is 50 mg/mL. For 8-Arg-3-S, the suggested solution concentration is 10 mg/mL. And for 2-Arg-6E-Cl (in the viscous state), the suggested concentration is 10 mg/mL. If needed, slightly

heat the solution in 60 degree isotherm heat bath to make sure all the polymers were completely dissolved. After that, vortex the solution for 5 seconds and let the polymer solution cool down to room temperature before making the Arg-PEA/DNA complex.

The Arg-PEA/ DNA complexes were prepared by adding the plasmid DNA solution with calculated volume to the freshly prepared Arg-PEA solutions to obtain systems with given DNA amount (1 μg /well for 24-well plate and 0.25 μg /well for 96-well plate) and weight ratios of Arg-PEA/DNA (for 2-Arg-3-S, the optimized ratio is 1000-3000; for 2-Arg-6E-CL, the ratio is 500-1000). 2-3 weight ratios are suggested for each polymer. The following are some details for the complex preparation: add the calculated amount of DNA solution to polymer solution, then perform immediate pipe up and down for 3-5 seconds after mixing the solutions (do not vortex or centrifuge), and then equilibrate solution in the cell culture hood for 20-30 minutes at room temperature (UV irradiation is suggested).

The RAW 264.7 macrophages cell lines were seeded at 30×10^3 /well in a 24-well plate or 5×10^3 /well in a 96-well plate 24-48 hours before transfection (70 % confluent at transfection). For 24-well plate, 1 μg COL (-772)/LUC or GFP DNA was formulated with the different Arg-PEA solutions at various weight ratios. For Superfect[®] control, 1 μg of plasmid DNA in 60 μL serum-free DMEM were supplemented with 5 μL (3 μg / μL) of the Superfect[®] (SF) solution according to manufacturer's recommendation. For Lipofectamine2000[®] control, 1 μg of plasmid DNA in 50 μL serum-free DMEM were supplemented with 2.5 μL of the Lipofectamine2000[®] solution in 50 μL serum-free DMEM according to manufacturer's recommendation. Immediately before transfection, cells were washed twice with 1X PBS, and then the 1 mL serum-free DMEM were added to each well.

Arg-PEA/DNA complex solution and control solutions were then added into each well immediately, piped up and down for 2-3 seconds. Cells were transfected for 3-4 h at 37 °C (5 % CO₂), and then the media was removed. After that, 0.5 mL of complete media for cell (follow the ATCC protocol) were added to each well and kept incubation. Cells were harvested for luciferase reading or GFP reading after 48 hours. Luciferase assay was performed according to Promega's recommendation. Briefly, cells from each well of a 24-well plate were lysed in 100 µL lysis buffer, transferred to a micro-tube, and then centrifuged at 10 000 g for 2 min. Supernatants were collected and analyzed for luciferase activity. In a typical experiment 20 µL of supernatant was added to luminometric tubes containing 100 µL of luciferase substrate (Promega). Light emission was measured with a Dual-luciferase detection system for a period of 5 sec. the relative light units/s was determined. Triplicates were used in each experiment.

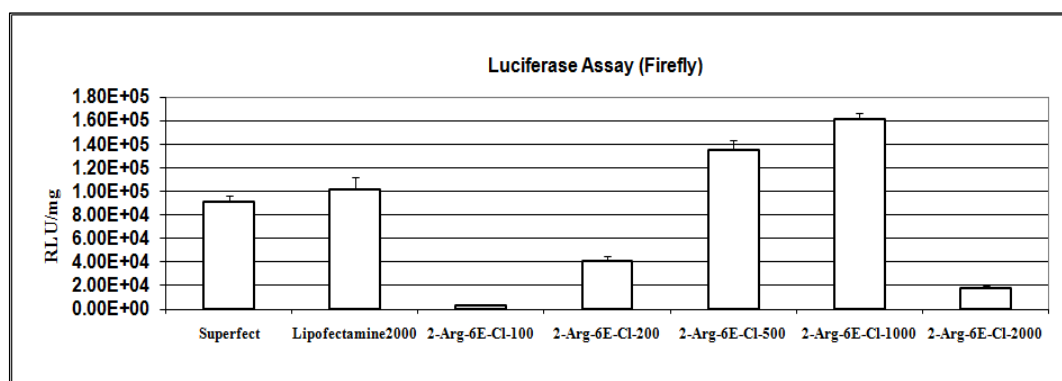


Figure 10 RAW 264.7 macrophages transfection efficiency evaluation of Arg-PEA/DNA complexes by firefly luciferase assay. Plasmid DNA used was COL (-772)/Luc. Lipofectamine2000[®] was used as the recommendations of Invitrogen[®] protocol. Various WRs of Arg-PEA to DNA were tested and the number after the Arg-PEAs was the corresponding WR.

A2.3.2 GFP assay

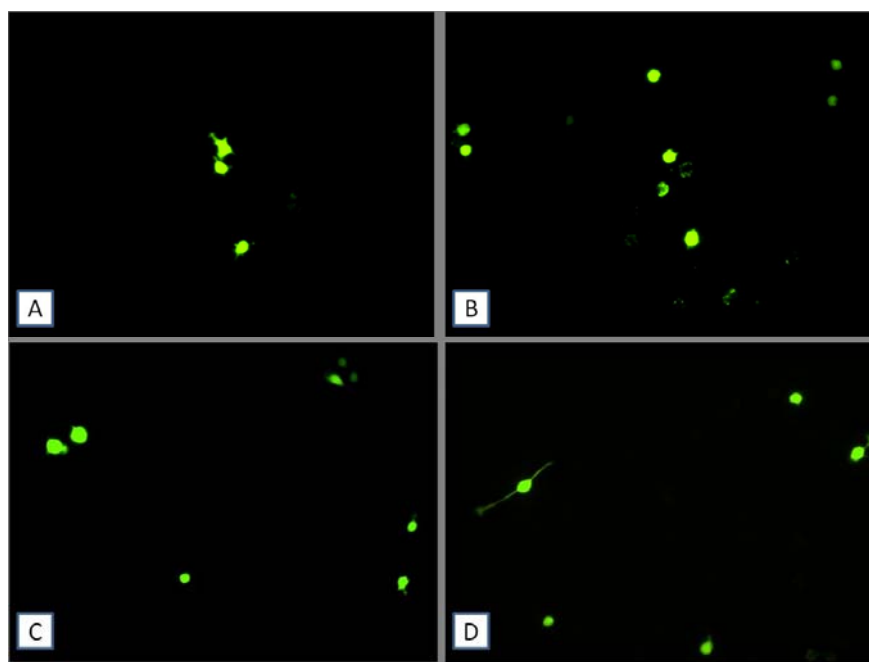


Figure 11 GFP transfection efficiency evaluation of RAW 264.7 macrophages under fluorescence microscope (10X). RAW 264.7 macrophages were transfected by (a) Superfect[®]; (b) Lipofectamine2000[®]; (c), 2-Arg-6E-Cl (WR=1,000); (d), 2-Arg-6E-Cl (WR=500). (4h treatment and GFP images were taken after 48h)

A2.3.3 MTT assay

The evaluation of the cytotoxicity of Arg-PEA/DNA complexes was performed by MTT assay. Cultured RAW 264.7 macrophages cell lines were seeded at an appropriate cell density concentration (10,000 cells/well) in 96-well plates and incubated overnight. Then the cells were treated with the freshly prepared aqueous

Arg-PEA/DNA solutions. Cells without material treated were used as blank control. Cells treated with Superfect[®]/DNA, Lipofectamine2000[®]/DNA, PEI/DNA and PLL/DNA were used for comparison. After 48 h incubation, 20 µL of MTT solution (5 mg/mL) was added to each well, followed by 4 h incubation at 37 °C, 5 % CO₂. The cell culture medium was then carefully removed and 200 µL of acidic isopropyl alcohol (with 0.1 M HCl) was added to dissolve the formed formazan crystal. The plate was slightly shaken for 20 mins to make sure the crystal dissolved completely. Absorbance (OD) was measured immediately at 570 nm (subtract background reading at 690 nm) using a microplate reader (VersaMax Tunable Microplate reader Molecular Devices, USA). The cell viability (%) was calculated according to the following equation:

$$\text{Viability (\%)} = (\text{OD}_{570(\text{sample})} - \text{OD}_{620(\text{sample})}) / (\text{OD}_{570(\text{control})} - \text{OD}_{620(\text{control})}) \times 100\%$$

Where the OD_{570(control)} represents the measurement from the wells treated with medium only and the OD_{570(sample)} from the wells treated with various polymer/plasmid DNA complexes. (Error bars represent mean + SEM, * P<0.05, number means the polymer/DNA weight ratio)

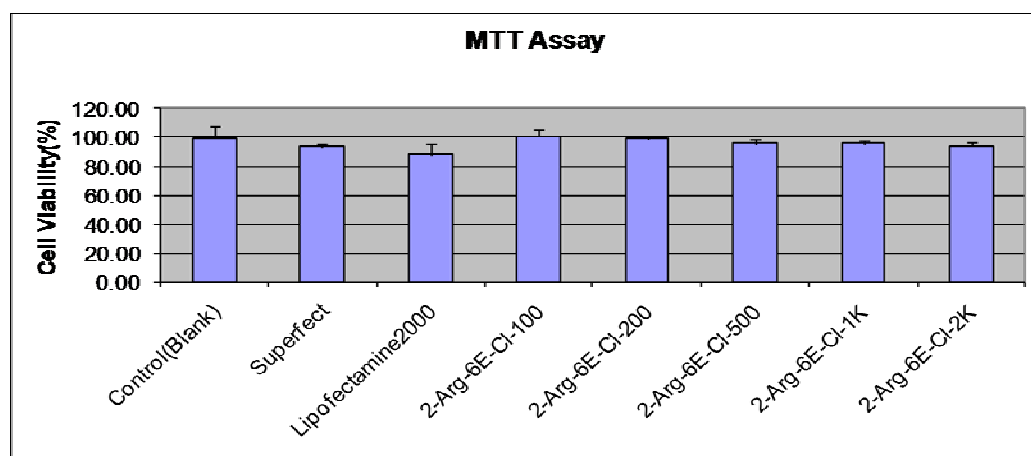


Figure 12 Cytotoxicity evaluation of Arg-PEA/DNA complexes by MTT assay. Control (blank) was cells only without any material treatment. Various WRs of Arg-PEA to DNA were tested. RAW 264.7 macrophages were treated by Arg-PEA/DNA complex for 4h. The number after the Arg-PEAs was the corresponding WR. Lipofectamine2000[®] and Superfect[®] were used as the recommendation of manufacturer protocol.

A2.3.4 Cell morphology (48 h, 10X) for RAW 264.7 macrophages cells

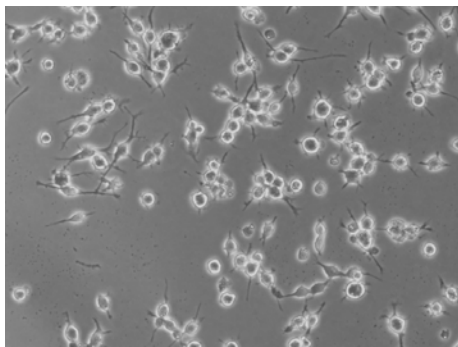


Figure 13 RAW 264.7 macrophages cell morphology under microscope (10X, 12 h treatment, picture taken after 48h). Cells were treated by 2-Arg-6E-Cl/DNA complex (WR=2000/1) at a concentration of 2 mg/mL for 4h

A2.4 Transfection and cytotoxicity evaluation of rat aortic fibroblast primary cell

Plasmid DNA was prepared and purified following the manufacturer's protocol. Arg-PEA 1X PBS buffer solution was freshly prepared in 1.5 mL microfuge tube right before the transfection test (start the transfection tests within 4 hours after making the Arg-PEA solution). And for 2-Arg-6E-Cl (in the viscous state), the suggested concentration is 10 mg/mL. If needed, slightly heat the solution in 60 degree isotherm heat bath to make sure all the polymers were completely dissolved. After that, vortex the solution for 5 seconds and let the polymer solution cool down to room temperature before making the Arg-PEA/DNA complex.

The Arg-PEA/ DNA complexes were prepared by adding the plasmid DNA solution with calculated volume to the freshly prepared Arg-PEA solutions to obtain systems with given DNA amount (1 $\mu\text{g}/\text{well}$ for 24-well plate and 0.25 $\mu\text{g}/\text{well}$ for 96-

well plate) and weight ratios of Arg-PEA/DNA (for 2-Arg-6E-CL, the ratio is 500-1000). 2-3 weight ratios are suggested for each polymer. The following are some details for the complex preparation: add the calculated amount of DNA solution to polymer solution, then perform immediate pipe up and down for 3-5 seconds after mixing the solutions (do not vortex or centrifuge), and then equilibrate solution in the cell culture hood for 20-30 minutes at room temperature (UV irradiation is suggested).

The rat aortic fibroblast primary cells were seeded at 30×10^3 /well in a 24-well plate or 5×10^3 /well in a 96-well plate 24-48 hours before transfection (70 % confluent at transfection). For 24-well plate, 1 μg COL (-772)/LUC or GFP DNA was formulated with the different Arg-PEA solutions at various weight ratios. For Superfect[®] control, 1 μg of plasmid DNA in 60 μL serum-free DMEM were supplemented with 5 μL (3 $\mu\text{g}/\mu\text{L}$) of the Superfect[®] (SF) solution according to manufacturer's recommendation. Immediately before transfection, cells were washed twice with 1X PBS, and then the 1 mL serum-free DMEM were added to each well. Arg-PEA/DNA complex solution and control solutions were then added into each well immediately, pipe up and down for 2-3 seconds. Cells were transfected for 3-4 h at 37 °C (5 % CO₂), and then the media was removed. After that, 0.5 mL of complete media for cell (follow the ATCC protocol) were added to each well and kept incubation. Cells were harvested for GFP reading after 48 hours.

A2.4.1 GFP assay

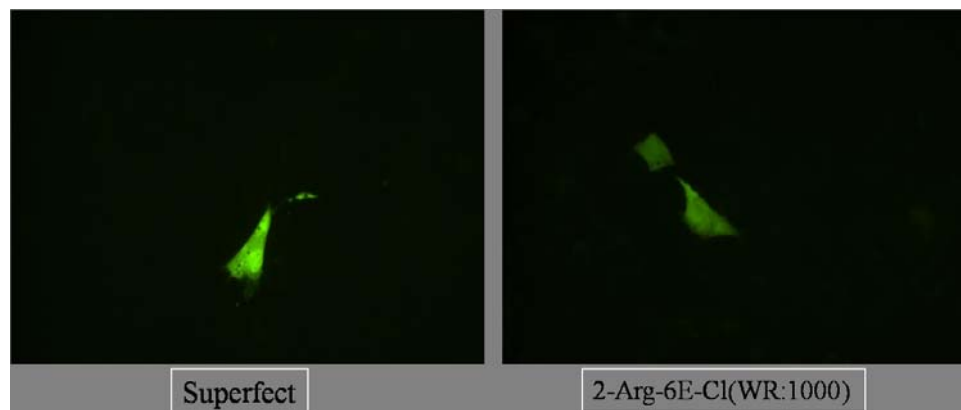


Figure 14 GFP transfection efficiency evaluation of rat aortic fibroblast primary cell under fluorescence microscope (10X). Rat aortic fibroblast primary cells were transfected by Superfect[®] and 2-Arg-6E-Cl (WR=1,000). (4h treatment and GFP images were taken after 48h)

A2.4.2 Cell morphology (48 h, 10X) for rat aortic fibroblast primary cell

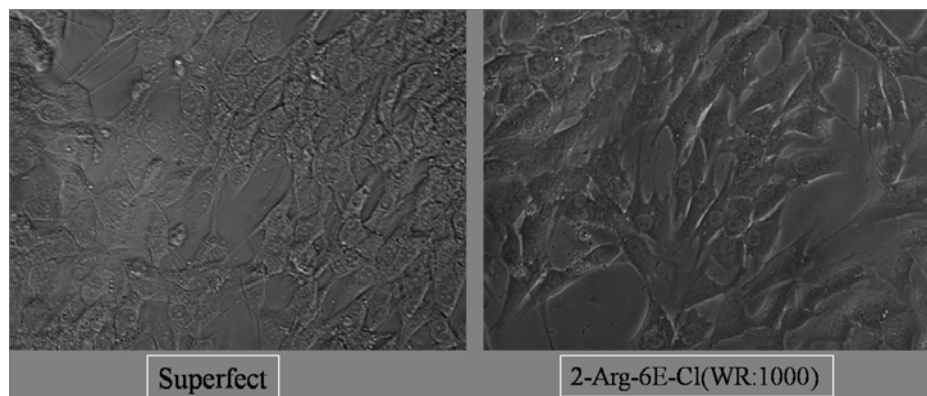


Figure 15 Rat aortic fibroblast primary cell morphology under microscope (10X, 12 h treatment, picture taken after 48h). Cells were treated by 2-Arg-6E-Cl/DNA complex (WR=1000/1) at a concentration of 2 mg/mL for 4h.

A2.5 Inflammation assay for Arg-PEA/DNA complex

The details of inflammation assay protocol exactly followed the manufacturer's protocol (TNF- α Mouse ELISA Kit of Invitrogen[®]). Figure 16 showed the evaluation of in vitro inflammation response of Arg-PEA/DNA complexes. The amount of nitrite (NO) produced from RAW macrophages was measured as the index of inflammation responses. Higher NO amount means the more severe inflammation response, while lower NO amount means the less inflammation response. The RAW macrophages were treated for 28 h. CpG DNA and Non CpG DNA were used as the controls. From Figure 16, it was found that compared with CpG DNA, the pure Arg-PEA polymers, including 2-Arg-3-S and 2-Arg-6E-Cl, caused much less inflammation response. And the NO production of Arg-PEA groups showed no significant difference from the non

CpG DNA group. Further investigation showed that compared with CpG DNA group, the Arg-PEA/ CpG DNA complex group caused less inflammation response, which indicated that when Arg-PEA formed complex with CpG DNA, the inflammation response of CpG DNA would be significantly reduced. Between the two Arg-PEA groups, 2-Arg-6E-Cl group showed much better inflammation response reduction performance than 2-Arg-3-S group. For the Arg-PEA/ Non CpG DNA complexes, they showed decreased inflammation response compared with Non CpG DNA, but the difference is not as significant as the CpG group.

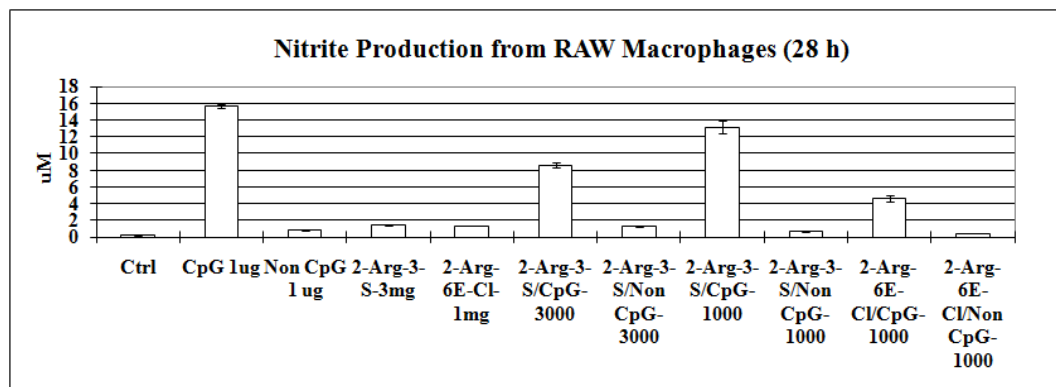


Figure 16 Nitrite production from RAW macrophages after 28 hrs' treatment. Ctrl: Control, no Arg-PEA or DNA added; CpG; the plasmid DNA causing severe inflammation response; Non CpG: the plasmid DNA causing no severe inflammation response; The DNA amount was fixed at 1 μ g/well for 24 well cell culture plate. The number after the Arg-PEA/DNA complex is the weight ratio of Arg-PEA to DNA

Appendix 3: Chapter 5 Arginine-based Poly (ester amide)s and Pluronic Diacrylate Cationic Hybrid Hydrogel for Tissue Engineering and Drug Delivery Applications

A3.1 BAEC and fibroblast cell attachment on hydrogel surface

Bovine endothelial aorta cells (BAECs) were purchased from VEC Technologies, kindly offered by Professor Cynthia Reinhart-King at Department of Biomedical Engineering of Cornell University. BAECs were cultured at 37 °C in 5 % CO₂ in Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10 % Fetal Clone III (HyClone, Logan, UT), and 1 % each of penicillin–streptomycin, MEM amino acids (Invitrogen, Carlsbad, CA), and MEM vitamins (Mediatech, Manassas, VA). BAECs were used from passages 8–12. Media was changed every 2 days. BAEC were grown to 70 % confluence before splitting or harvesting. Cell culture plates were coated with 2 wt% gelatin aqueous solution before using.

The cell attachment and proliferation on the Arg-UPEA/PluronicDA/PEGDA hybrid hydrogel surfaces was evaluated by cell morphology. Pure PluronicDA or PluronicDA/PEGDA hybrid hydrogel was selected as the hydrogel control and the cell culture plate without any treatment was used as the negative control. The cells used for this study were BAEC cells. The purified hydrogels were cut into round shape with the diameter that just filled the well of 24-well cell culture plates. Before being put into the 24-well cell culture plates, the hydrogels were sterilized under UV light (from the cell culture hood) for 1 h. After that, the hydrogels were washed twice by PBS buffer and cell culture media. Then, the hydrogels were placed into the wells of the cell culture plate and fixed by sterilized rubber ring which has the same diameter as the well of cell culture plate. BAEC cells were seeded at an appropriate cell density

(10,000 cells/well) and incubated overnight. After 48 h incubation, the cell attachment and proliferation on the hydrogel surface was record by an optical microscope.

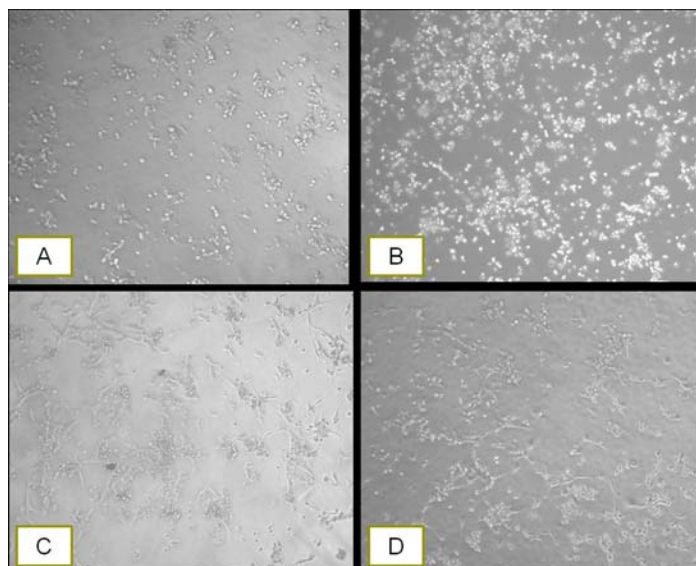


Figure 17 Representative micrographs of BAEC cells after 48 hrs' culture on the hydrogel surface.10x. (A) F68-PEG700 (3/1, w/w); (B) F127-PEG700 (3/1, w/w); (C) F127-PEG700-2-UArg-2-S(3/1/1, w/w/w); (D) F127-PEG700-2-UArg-2-S(4.5/1.5/4, w/w/w).

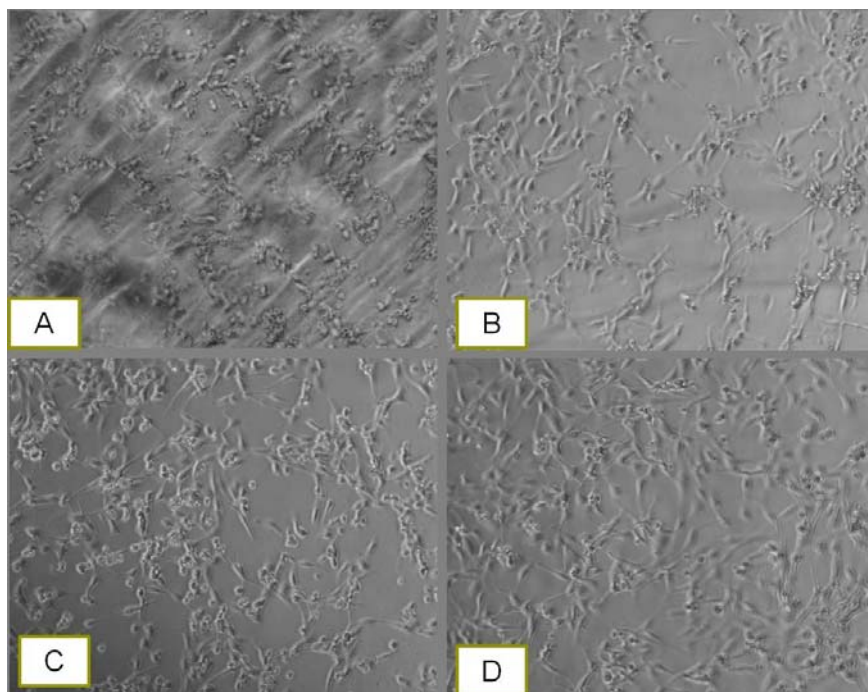


Figure 18 Representative micrographs of BAEC cells after 48 hrs' culture on the collagen treated hydrogel surface. Hydrogel was treated with collagen by soaking in 2% collagen water solution for 5 mins. 10x. (A) F68-PEG700 (3/1, w/w) ;(B) F127-PEG700 (3/1, w/w); (C) F127-PEG700-2-UArg-2-S(3/1/1, w/w/w); (D) F127-PEG700-2-UArg-2-S(4.5/1.5/4, w/w/w).

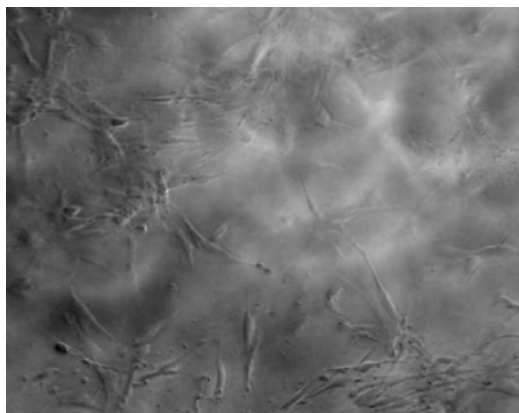


Figure 19 Representative micrographs of Human Detroit 539 fibroblast cells after 48 hrs' culture on the hydrogel (F68-PEG700 (3:1/w: w)) surface. 10x.

Appendix 4: Chapter 6-Poly (ester amide)-b-Poly (ϵ -caprolactone): Synthesis, Characterization, Formulation, and *In Vitro* Cellular Response

A4.1 Materials

L-Phenylalanine (L-Phe), *p*-toluenesulfonic acid monohydrate (TosOH·H₂O), adipoyl chloride, sebacoyl chloride, 1, 4-butanediol, 1,6-hexanediol and *p*-nitrophenol were all purchased from Alfa Aesar (Ward Hill, MA) and used without further purification. Triethylamine from Fisher Scientific (Fairlawn, NJ) was dried by refluxing with calcium hydride, and then distilled before use. Solvents like toluene, ethyl acetate, acetone, 2-propanol; *N*, *N*-dimethylacetamide (DMA) and dimethyl sulfoxide (DMSO) were purchased from VWR Scientific (West Chester, PA) and were purified by standard methods before use. Other chemicals and reagents if not otherwise specified were purchased from Sigma (St. Louis, MO).

A4.2 Synthesis of PEAs

The preparation of PEAs involved the following three basic steps: (1) the synthesis of monomer **I**: di-*p*-nitrophenyl esters of dicarboxylic acids; (2) the synthesis of monomer **II**: di-*p*-toluenesulfonic acid salts of bis-L-phenylalanine esters; (3) the synthesis of PEAs via the solution polycondensation of monomers **I** and **II**. For PEA monomers, the synthesis steps were exactly followed the reported methods. About the synthesis of PEAs, different polymerization conditions were evaluated. In the report, we mainly focused on the effects of molar ratios of monomers ($r=\text{I/II}$) and polymerization time. Other conditions and steps were exactly followed the reported references.

A4.3 Material characterization

The chemical structures of the synthesized monomers and polymers were characterized with standard chemical methods. For Fourier transform infrared (FTIR) characterization, the samples were ground into powders and mixed with KBr at a sample/KBr ratio of 1:10 (w/w). FTIR spectra were then obtained with a PerkinElmer (Madison, WI) Nicolet Magana 560 FTIR spectrometer with Omnic software for data acquisition and analysis. NMR spectra were recorded with a Varian (Palo Alto, CA) Unity Inova 400-MHz spectrometer operating at 400 and 100 MHz for ^1H and ^{13}C NMR, respectively. Deuterated chloroform (CDCl_3 -*d*1; Cambridge Isotope Laboratories, Andover, MA; for NA and NS) with tetramethylsilane as an internal standard or deuterated dimethyl sulfoxide ($\text{DMSO-}d_6$; Cambridge Isotope Laboratories; for NA, NS, PB and All polymers) was used as the solvent. The number-average molecular weight (M_n), weight-average molecular weight (M_w), and molecular weight distribution (MWD) of the synthesized polymers were determined with a model 510 gel permeation chromatograph (Waters Associates, Inc., Milford, United States) equipped with a high-pressure liquid chromatography pump, a Waters 486 UV detector, and a Waters 2410 differential refractive index detector. Tetrahydrofuran (THF) was used as the eluent (1.0 mL/min). The columns were calibrated with polystyrene standards with a narrow MWD.

A4.4 Results and discussion

The following two PEAs were selected for this study: 4-Phe-4 and 8-Phe-4. First, the two polymers were investigated for the relationship between M_n and polymerization time (h) at the monomer molar ratio of **I** /**II** ($r=\text{NS/PB}$) of 1.0. Figure

20 showed the M_n -Time curve of polymerization of 4-Phe-4 and Figure 21 showed the M_n -Time curve of polymerization of 8-Phe-4. Both figures indicated that at the beginning several hours, the M_n of PEA significantly increased with a very fast speed. After that, the M_n of PEA would slowly increase for a few more hours. The whole M_n increasing time would be around 10-15 h. Then the M_n of PEA would be at a constant value, which is around 20-30 Kg/mol, depending on the PEA type. For 4-Phe-4, it's around 22 Kg/mol, while for 8-Phe-4, it's around 25 Kg/mol.

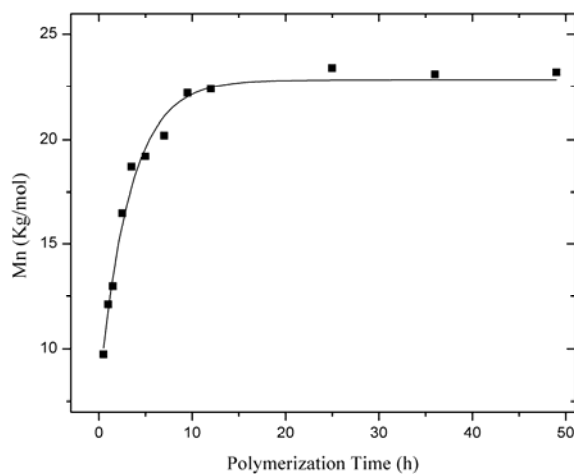


Figure 20 M_n -Time curve of polymerization of 4-Phe-4 with $r=1.00$

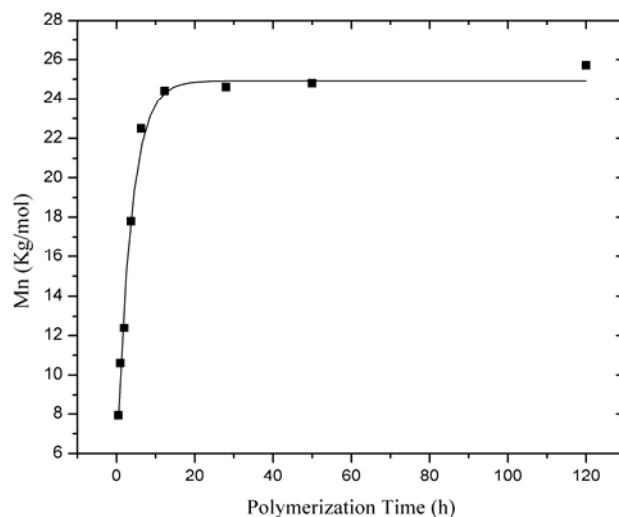


Figure 21 M_n -Time curve of polymerization of 8-Phe-4 with $r=1.00$

Then we continued to investigate the 8-Phe-4 for the relationship between M_n and polymerization time (h) at the monomer molar ratios of **I** / **II** ($r=NS/PB$) other than 1.0. Two molar ratios, 0.9 and 0.8 were selected. Figure 22 and 23 showed the M_n -Time curve of polymerization of 8-Phe-4 with molar ratios of 0.9 and 0.8, respectively. The figures showed that within the beginning 1 hour, the M_n of PEA almost immediately reached a constant value, depending on the molar ratio. For $r=0.90$, it's around 7 Kg/mol, while for $r=0.80$, it's around 4 Kg/mol. After that, the PEA M_n would not change. The M_n increasing trend and final M_n value of PEAs were reproducible.

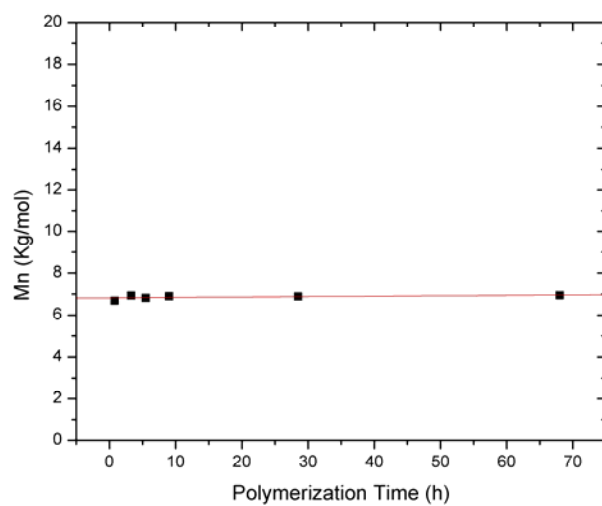


Figure 22 M_n -Time curve of polymerization of 8-Phe-4 with $r=0.90$

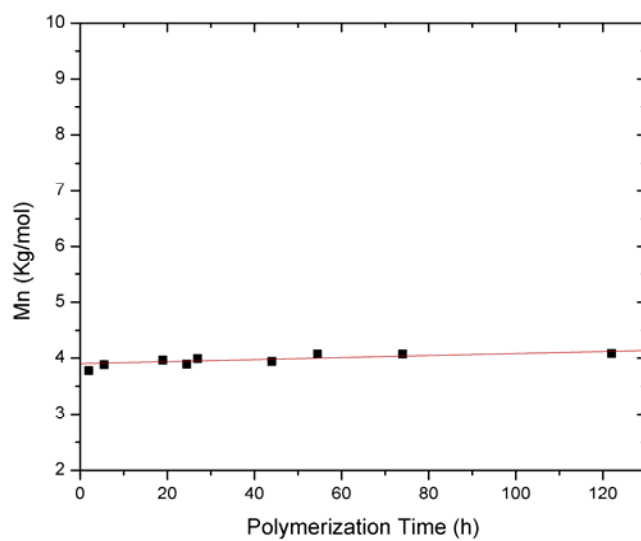


Figure 23 M_n -Time curve of polymerization of 8-Phe-4 with $r=0.80$

Figure 24 showed the M_n -Time curve of polymerization of 8-Phe-4-b-PCL with a weight ratio of 1 to 6 for 8-Phe-4 to ϵ -CL and the M_n of 8-Phe-4 is around 4,000

Kg/mol. And Figure 25 showed the M_n -Time curve of polymerization of 8-Phe-4-b-PCL with a weight ratio of 1 to 6 for 8-Phe-4 to ϵ -CL and the M_n of 8-Phe-4 is around 7,000 Kg/mol. Both figures showed that at the beginning several hours, the M_n of PEA-*b*-PCL significantly increased with a very fast speed. After that, the M_n of PEA-*b*-PCL would slowly increase for a few more hours. The whole M_n increasing time would be around 15-25 h. Then the M_n of PEA would be at a constant value, which depends on the M_n of PEA. Higher M_n of PEA would cause higher M_n of PEA-*b*-PCL.

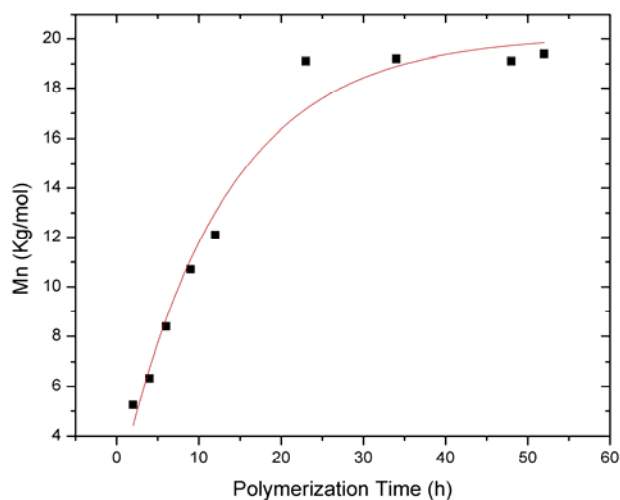


Figure 24 M_n -Time curve of polymerization of 8-Phe-4-b-PCL (8-Phe-4 with M_n of 4k, 8-Phe-4/ ϵ -CL=1/6, w/w)

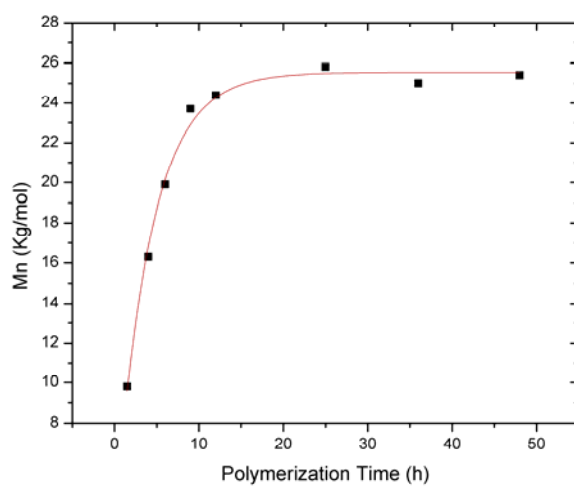


Figure 25 M_n -Time curve of polymerization of 8-Phe-4-b-PCL (8-Phe-4 with M_n of 7k, 8-Phe-4/ ϵ -CL=1/6, w/w)